BRITISH JOURNAL OF CANCER

VOL. XI MARCH, 1957 NO. 1

MORTALITY RATES FOR CANCER OF URINARY BLADDER IN VARIOUS COUNTRIES

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Received for publication November 17, 1956

THE recent demonstration of an increase in incidence among men in Copenhagen of papillomata and carcinomata of the urinary bladder, reported by two of us (Clemmesen and Nielsen, 1956) and illustrated in Fig. 1, has induced us to compare as far as possible the figures for this disease from a number of countries. It is realized by the authors that a real and final comparison of such figures can only be undertaken through direct collaboration between experts from countries concerned, so that the present publication will only serve to draw the attention of such experts to the possibility that the increase observed in Copenhagen may apply also to other areas.

At present such comparison will have to be limited to mortality rates because incidence figures are only available for a few countries. Many countries do not even report specific mortality figures for tumours of the bladder, but this difficulty has been overcome through the courtesy of Dr. Y. Biraud, The Epidemiological and Statistical Division of World Health Organization, Geneva, who has kindly placed the basic figures for this study at our disposal.

A more important limitation of our study is that these figures will refer only to cancer, which exposes them to possible errors in two ways: First, papillomata will not be included, which lesions, as far as etiology studies are concerned, are probably as important as the fully developed cancers into which most of them finally develop. Probably papillomata represent a larger proportion of bladder tumours in areas where the latter are increasing in frequency than where this is not the case. Second, it is reasonable to assume that cure is more easy to attain in cases of papilloma, so that differences between countries in the proportion of papillomata as well as in the efficiency of therapy may both influence our figures.

On the other hand the Copenhagen figures suggest that an increase may be observed in cancer cases alone, and in order to give an idea of the interrelation between morbidity and mortality figures as far as Denmark is concerned, we have given the age distribution of cases as well as of deaths for all Denmark in Fig. 2 and Table I together with morbidity rates at various ages for men in Copenhagen for the two subsequent five-year periods concerned. Maybe other areas with rates similar to those for all Denmark may comprise subdivisions in which an

increase in cases similar to the increase observed in Copenhagen may appear on more detailed study, thus facilitating investigations into aetiology.

Fig. 3 to 13 illustrate mortality rates for vesical cancer among men at various ages per 100,000 inhabitants. The basic figures are given in Table II, and population figures have been taken from World Health Organization's *Annual Epidemiological and Vital Statistics* (1956).

It should be mentioned that differences in age groups beyond 65 should be taken with some reservation, first because the populations in these age groups are limited so that relatively few cases may account for the differences, and second

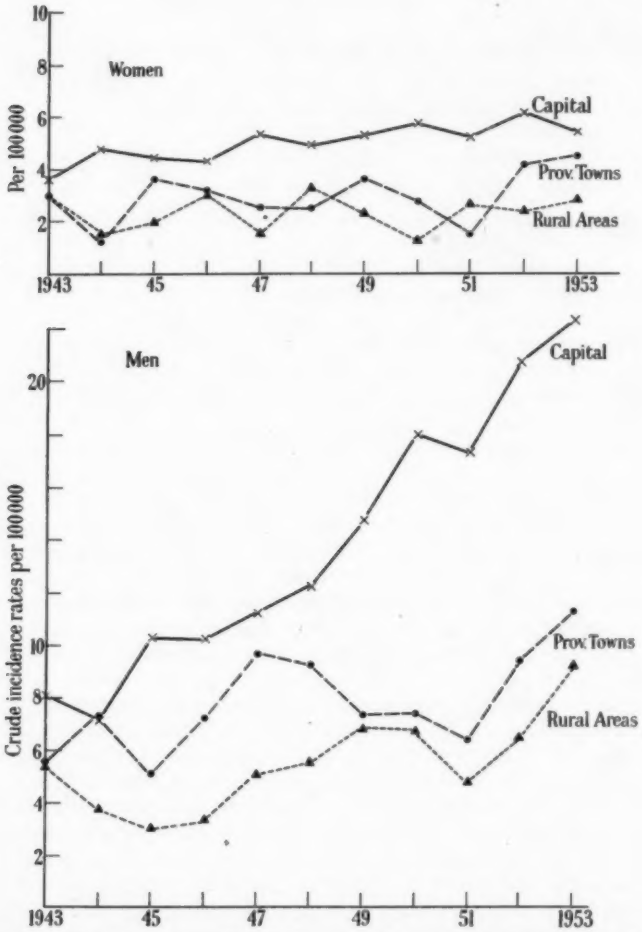


FIG. 1.—Crude incidence rates per 100,000 for cases of cancer and of papilloma of urinary bladder, ureter and urethra among women and men in Danish capital, provincial towns and rural areas from 1943–53.

because the last point has been determined for the groups aged over 75 as a whole. We have not deemed it advisable for this orientating study to make any calculations of standard error.

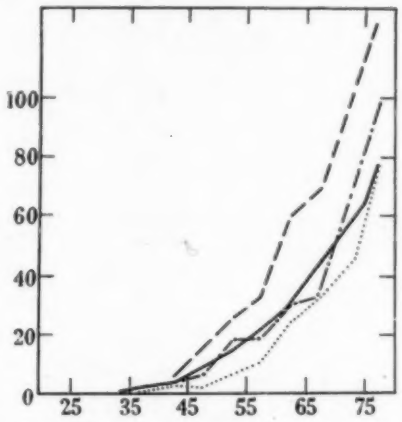


FIG. 2.—Mortality and morbidity rates at various ages for tumours of urinary bladder among men in Denmark and Denmark's capital.

..... Mortality rates for men, Denmark, 1952–53, cancer of bladder and urethra (W.H.O. Code No. 181).

———— Morbidity rates for men, Denmark, 1948–52, cancer and papillomata of bladder, ureter, urethra.

·—·—·—· Morbidity rates for men, capital, 1943–47, cancer and papillomata of bladder, ureter, urethra.

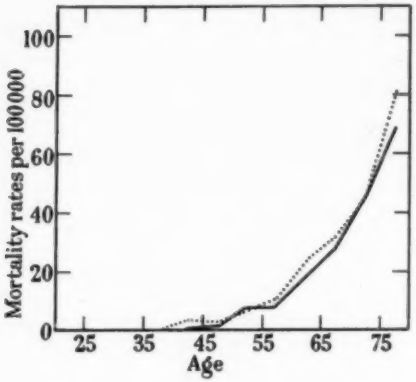
— — — — Morbidity rates for men, capital, 1948–52, cancer and papillomata of bladder, ureter, urethra.

It appears from the diagrams that the age distribution of deaths per 100,000 follows approximately the same course as the Danish curve for the following countries: Australia, Canada, England and Wales, Italy, Netherlands, Scotland,

TABLE I.—*Carcinoma and Papilloma of Urinary Bladder, Ureter and Urethra*

Morbidity rates per 100,000 for men at various ages

Age	Greater Copenhagen, 1943–47	Greater Copenhagen, 1948–52	All Denmark, 1948–52
24	0·0	0·0	0·0
25–29	2·1	0·6	0·4
30–34	1·0	1·7	0·9
35–39	1·1	3·2	1·1
40–44	2·4	5·0	3·6
45–49	8·0	15·6	8·1
50–54	19·7	27·1	14·5
55–59	20·8	37·2	21·2
60–64	31·1	64·3	30·1
65–69	35·5	73·6	42·5
70–74	69·5	99·8	57·9
75–	100·5	126·0	75·7



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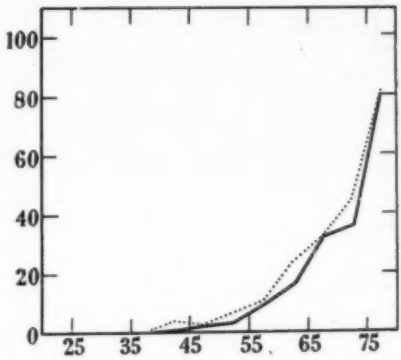

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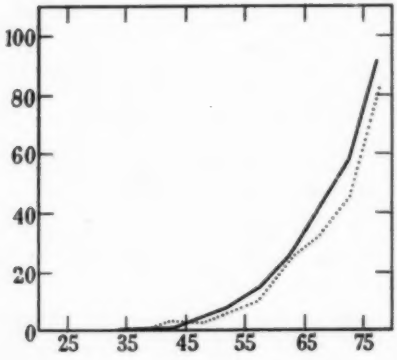

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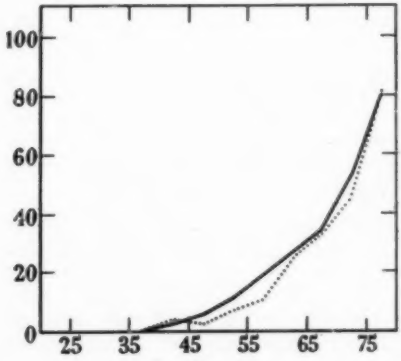

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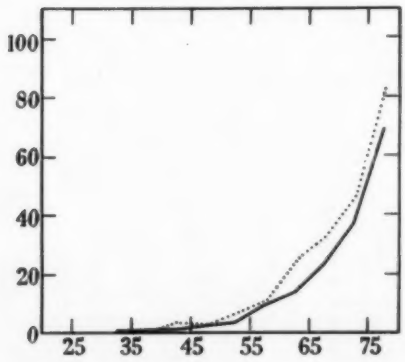

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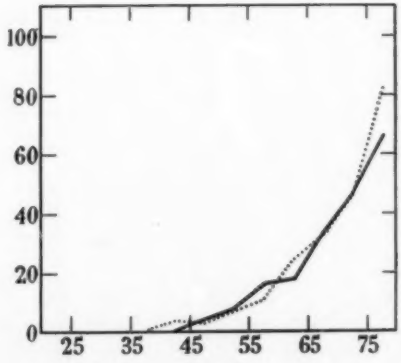

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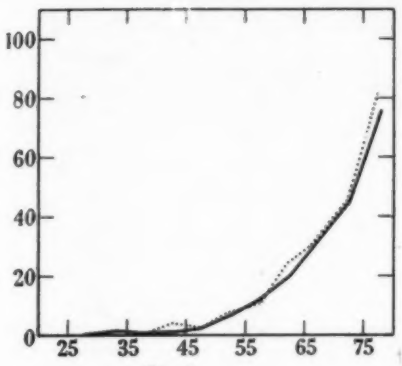

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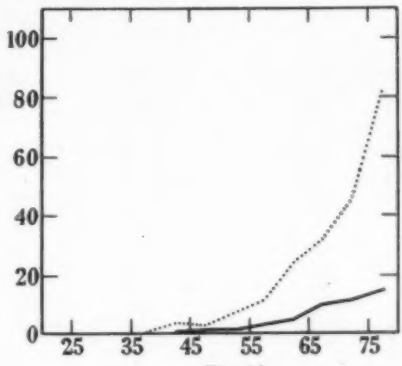

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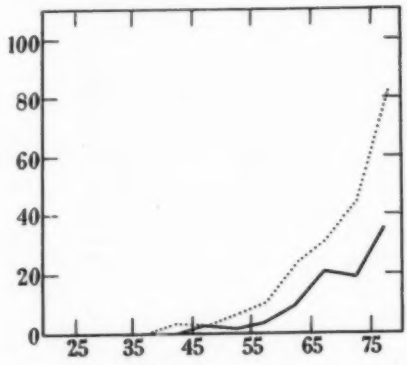

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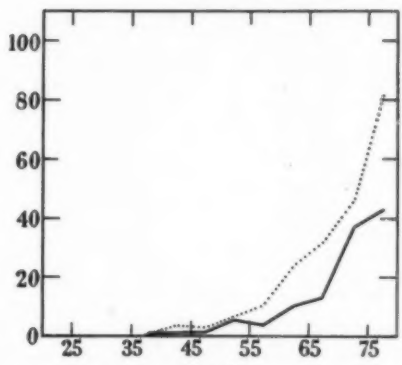

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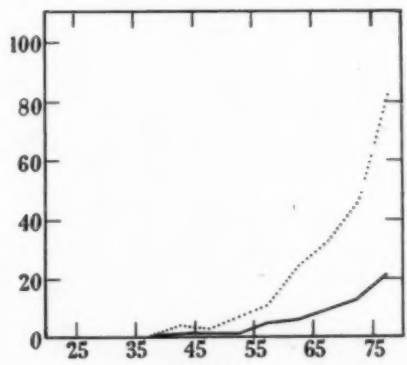

FIG. 13.

FIG. 3–13.—Mortality rates at various ages for cancer of urinary bladder and urethra (W.H.O. Code No. 181) among men in various countries. The dotted line represents the curve for Denmark. Fig. 3.—Australia, 1952–53. Fig. 4.—Canada, 1952–53. Fig. 5.—England and Wales, 1952–53. Fig. 6.—Italy, 1952–53. Fig. 7.—Netherlands, 1952–53. Fig. 8.—Scotland, 1952–53. Fig. 9.—U.S.A., 1952–53. Fig. 10.—Japan, 1952–53. Fig. 11.—New Zealand, 1952–53. Fig. 12.—Norway, 1952–53. Fig. 13.—Sweden, 1952–53.

TABLE II*a*

Men	Australia, 1952–53. Deaths	Canada, 1952–53. Deaths	Denmark, 1952–53. Deaths	England and Wales, 1952–53. Deaths
24	3	3	0	4
25–29	2	0	0	1
30–34	0	5	0	3
35–39	0	4	0	15
40–44	5	5	11	37
45–49	10	18	7	144
50–54	37	27	17	230
55–59	33	59	24	334
60–64	66	90	43	489
65–69	75	149	48	639
70–74	82	124	51	695
75–	127	281	101	1123
Total	440	765	302	3714
Women				
24	0	2	0	6
25–29	0	1	0	2
30–34	0	1	0	4
35–39	2	1	3	4
40–44	1	3	1	28
45–49	6	8	3	51
50–54	8	18	2	81
55–59	11	12	7	120
60–64	22	26	15	153
65–69	22	47	18	211
70–74	38	55	19	268
75–	71	148	44	615
Total	181	322	112	1543

TABLE II*b*

Men	Italy, 1952–53. Deaths	Japan, 1952–53. Deaths	Netherlands, 1952–53. Deaths	New Zealand, 1952–53. Deaths
24	[illegible]	3	1	0
25–29	3	1	0	0
30–34	4	2	1	0
35–39	16	1	2	0
40–44	39	7	4	0
45–49	68	15	15	3
50–54	135	21	22	2
55–59	172	39	43	3
60–64	210	54	52	7
65–69	232	79	70	14
70–74	269	63	89	10
75–	427	63	177	19
Total	1586	348	476	58
Women				
24	2	3	1	0
25–29	1	0	0	0
30–34	2	3	1	0
35–39	6	2	3	0
40–44	6	9	4	0
45–49	15	8	5	1
50–54	38	19	10	2
55–59	51	41	11	1
60–64	76	49	22	2
65–69	108	44	27	1
70–74	94	40	31	4
75–	143	45	79	10
Total	542	263	194	21

TABLE IIc

Men	Norway, 1952–53. Deaths	Scotland, 1952–53. Deaths	Sweden, 1952–53. Deaths	United States, 1952–53. Deaths
24	0	2	0	22
25–29	0	0	0	4
30–34	0	2	0	8
35–39	0	2	2	35
40–44	1	1	3	118
45–49	1	13	3	248
50–54	5	23	2	504
55–59	4	39	17	918
60–64	7	36	16	1308
65–69	7	53	23	1626
70–74	15	61	25	1619
75–	23	91	49	2880
Total	63	323	140	9290
Women				
24	0	0	0	6
25–29	0	0	0	7
30–34	0	0	0	11
35–39	0	2	1	47
40–44	2	3	2	71
45–49	0	7	1	140
50–54	2	7	4	224
55–59	3	11	2	306
60–64	5	18	8	426
65–69	9	27	9	625
70–74	4	33	9	766
75–	27	47	27	1732
Total	52	155	63	4361

and particularly U.S.A., while on the contrary Japan, New Zealand, Norway and Sweden show rates considerably lower than the Danish. This seems particularly striking for Norway and Sweden which in so many ways are similar to Denmark.

SUMMARY

A crude comparison has been made of mortality rates at various ages for cancer of the urinary bladder among men from the following countries : Australia, Canada, Denmark, England and Wales, Italy, Japan, Netherlands, New Zealand, Norway, Scotland, Sweden and U.S.A.

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PITUITARY DESTRUCTION BY INJECTION OF RADIOACTIVE SUBSTANCE AND SECTION OF THE PITUITARY STALK FOR ADVANCED CANCER

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Received for publication January 30, 1957

TRANSFRONTAL hypophysectomy is an established surgical procedure in the treatment of advanced cancer. Results of this operation have been reported by Perrault, Le Beau, Klotz, Sicard and Clavel (1952), Shimkin, Boldrey, Kelly, Beirman, Ortega and Naffziger (1952), Luft and Olivecrona (1953) and by Pearson Ray, Harrold, West, Li, Maclean and Lipsett (1956).

Luft and Olivecrona (1953) performed hypophysectomy in 15 cases of malignant disease. Twelve cases suffered from carcinoma of the breast, one from chorion carcinoma, one from carcinoma of the kidney, and one from carcinoma of the prostate. They showed that when hypophysectomy is complete a remarkable regression of the carcinoma may occur in cases of metastatic breast cancer. There were no operative deaths in this series.

Pearson *et al.* (1956) have reported a series of 79 patients with advanced malignant disease on whom hypophysectomy had been performed from 1953–55. There were 11 operative deaths in the whole series. Forty-one patients with metastatic breast cancer could be evaluated and 21 of these obtained objective remission.

All the available evidence indicates that only those cases of breast cancer which are hormone dependent benefit from removal of the pituitary. The present difficulty in selecting patients with breast cancer for operation is to determine those whose growths are in fact hormone dependent.

Two possible tests are available. In one (Pearson *et al.*, 1956) oestrogens are given and the effect on the mammary cancer noted, an increase in activity indicates that the growth is influenced by oestrogens and that hypophysectomy might be of value. The second test is the estimation of the mammatrophins in the urine (Hadfield, 1956). Further work on these methods is awaited with interest. It should be noted however that while patients who have had a favourable response to oophorectomy or to androgen therapy are likely to have a favourable response to hypophysectomy, failure to respond to androgen or oestrogen therapy does not necessarily mean that the patient will fail to respond to hypophysectomy.

In view of the reported results of hypophysectomy in advanced cancer, it was decided to attempt to destroy the pituitary gland by local irradiation in cases of malignant disease where the tumour was likely to be hormone dependent. Chromic phosphate was injected into the gland, the phosphorus being in the form of the radioactive isotope P^{32}. This method was described by Rothenberg, Jaffe, Putnam and Simkin (1955). It appeared a less severe procedure than hypophysectomy in a seriously ill patient.

Radioactive colloidal chromic phosphate seemed theoretically an ideal substance for pituitary ablation by radiation. It is precipitated upon injection and

remains at the injection site; P^{32} emits beta radiation only with a maximum effective penetration of 0·8 mm. in body tissue; it has a half-life of 14 days which is very convenient when planning the operation. Radioactive gold does not remain at the injection site and it has a half-life of 3½ days, thus like radioactive yttrium (half-life 61 hours), it has to be obtained immediately before operation to get a sufficiently high dose of radioactive substance in a small volume of the injection material.

Pituitary Destruction by Injection of Radioactive Chromic Phosphate

Between October 1955 and June 1956, 5 cases of advanced carcinoma of the breast with widespread metastases and 1 case of malignant melanoma with metastases were treated by injection of radioactive chromic phosphate into the pituitary gland. These 6 cases form Group I (Table I). The effectiveness of the procedure was assessed by endocrine and electrolyte changes.

TABLE I.—*Summary of Cases Treated for Advanced Carcinoma*

Group I

Cases treated by injection of radioactive chromic phosphate

Case	Age	Sex	Site of primary tumour	Post-operative survival
1	42	F.	Breast	Died at 7 weeks
2	28	,,	Skin (malignant melanoma)	,, ,, 26 ,,
3	36	,,	Breast	,, ,, 10 ,,
*4	47	,,	,,	,, ,, 30 ,,
5	54	,,	,,	,, ,, 29 ,,
6	40	,,	,,	,, ,, 22 ,,

* Stalk section carried out 23 weeks after injection.

Group II

Cases treated by injection of radioactive material, and section of pituitary stalk

Case	Age	Sex	Site of primary tumour	Post-operative survival
4	47	F.	Breast	Died at 30 weeks
7	50	,,	,,	,, ,, 4 ,,
8	52	,,	,,	,, ,, 9 days.

In Groups I and II operation produced no appreciable effect on the tumour.

Group III

Cases treated by section of the pituitary stalk

Case	Age	Sex	Site of primary tumour	Effect on tumour	Post-operative survival
9	57	F.	Breast	Relief of bone pain	Alive at 21 weeks.
10	58	,,	,,	,, ,,	Died at 14 ,,
11	42	,,	,,	Regression of cutaneous and pleural deposits	Alive at 13 ,,
12	63	M.	Prostate	Marked fall in acid phosphatase	Alive at 12 ,,
13	46	F.	Breast	No change	Died at 10 ,,
14	42	,,	,,	,, ,,	Alive at 5 ,,

Investigations

The following tests of pituitary function were carried out before and after operation: estimation of the basal metabolic rate, the radioactive iodine uptake and excretion, the urinary 17 ketosteroids (17 KS) and glucocorticoids (17 OH). The change in excretion of the glucocorticoids was found to be of more significance than the change in the ketosteroids in assessment of adrenocortical function, as ketosteroid output is always low in seriously ill patients. A complete radiological survey was undertaken and estimations of the 24-hourly urinary calcium excretion were made. A full blood count was done and the blood urea, serum cholesterol, blood sugar, plasma proteins and electrophoretic pattern and serum electrolytes were estimated. The most helpful of all these tests were found to be the radioactive iodine uptake and excretion and the glucocorticoid estimation.

Operative procedure

This has been previously described (Davies, 1956). In summary, a coronal scalp incision is made, the flap being turned down over the forehead and eyes. A trephine opening 1½-in. diameter is made above the superior orbital ridge, the frontal lobe is elevated, the olfactory tract is cut and the pituitary stalk is seen below the optic chiasm. After injection of the radioactive material the bone disc is replaced and the scalp sutured in the usual way. The amount of radioactive material injected varied from 3–8 millicuries in 0·2–0·4 ml. of solution.

Post-operative complications

All patients complained of headache and a feeling of fullness behind the eyes for a few days, aspirin or codeine tablets were sufficient for the relief of these symptoms. Somnolence was noted in all patients but in only 1, Case 6, was cortisone necessary for relief. One patient developed a temporary right temporal field defect which cleared up in 10 days. Another patient complained of mistiness of vision which disappeared in 14 days.

Results

In the first 5 cases there was no clinical nor endocrine evidence of effective pituitary destruction. In Case 6 however there was profound disturbance soon after operation (Fig. 1). In this case there had been some haemorrhage in the pituitary region at operation and it was thought that the pituitary stalk might have been damaged whilst controlling this bleeding.

Autopsy was possible only in Case 2 of this group. Widespread tumour deposits were found in this case. The pituitary gland showed partial destruction of the epithelial elements (Fig. 2) but viable glandular tissue remained. The hypothalamus showed nodules of tumour cells but no gross parenchymal damage (Fig. 3, 4). The thyroid and suprarenals though extensively infiltrated by carcinoma showed no gross damage to secretory tissue.

In view of these findings and the absence of endocrine changes in life in the cases other than Case 6, it was decided to combine injection of radioactive chromic phosphate with deliberate destruction of the pituitary stalk. Three cases were treated in this manner and form Group II (Table I). Pre- and post-operative

investigations were done as before. The technique of the combined operation was as described above but after the gland had been injected the pituitary stalk was divided close to the gland and the ends cauterised. (Fig 5).

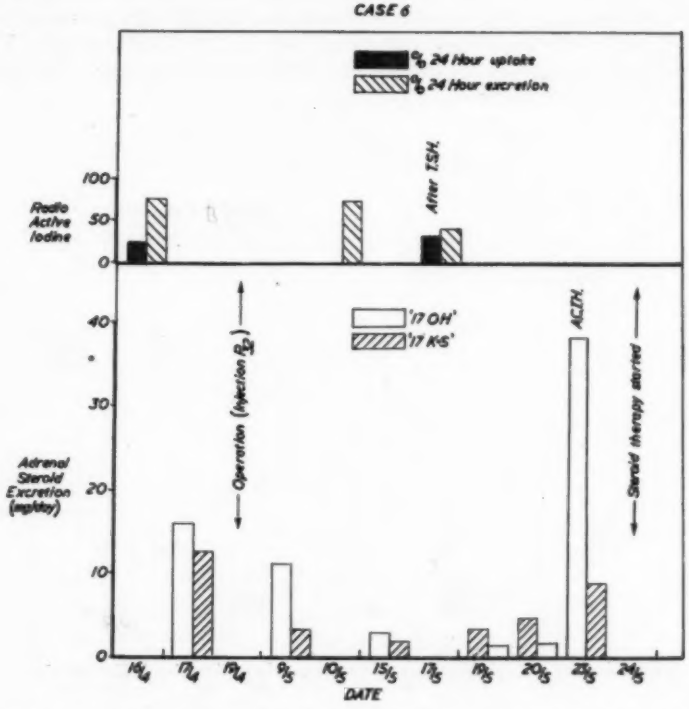


FIG. 1.—Case 6. Post-operative fall in radioactive iodine uptake and fall in "17 OH" excretion. Reversal of these changes after thyrotrophic and adrenotrophic hormone is also shown.

Pituitary Destruction by Injection Combined with Stalk Section

The first two cases treated by this combined method showed marked endocrine changes (Fig. 6). As Case 4, treated earlier by injection only, still showed no endocrine disturbance, nor was there any alleviation of symptoms from malignant disease, she was operated upon again and stalk section was performed. Immediately there were profound endocrine changes (Fig. 7). There was however no appreciable change in the patient's extensive malignant disease, the local skin deposits practically surrounded the chest.

Autopsy was possible in Cases 7 and 8 treated by the combined method. In Case 7 gross pituitary destruction was found though a small island of viable epithelial cells remained (Fig. 8). The hypothalamus in this case showed very

marked parenchymal damage which was thought to be due to ascending thrombosis from the cauterised, divided pituitary stalk (Fig. 9). Small groups of tumour cells were also seen in the hypothalamus (Fig. 10). The thyroid showed no gross histological abnormality; the suprarenal glands had been removed previously. In Case 8, despite the marked endocrine changes found in life, autopsy showed only partial destruction of the pituitary epithelial tissue, no gross hypothalamic damage, but complete necrosis of the distal part of the pituitary stalk (Fig. 11). The thyroid and suprarenal cortex showed degenerative changes and extensive infiltration by carcinoma cells.

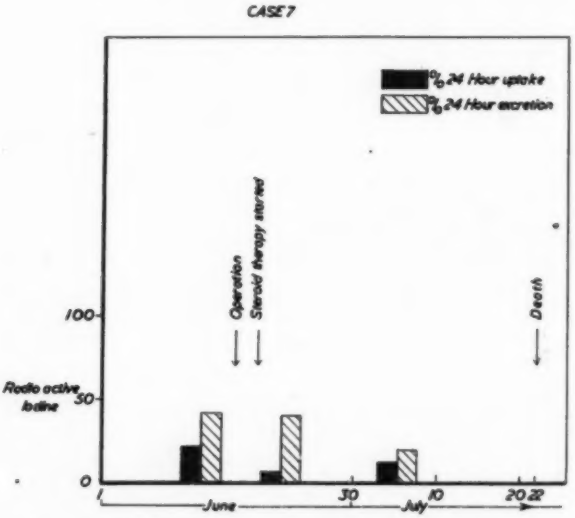


FIG. 6.—Case 7. To show post-operative fall in radioactive iodine uptake.

These results suggested that destruction of the pituitary stalk produced profound endocrine disturbance despite incomplete destruction of the pituitary glandular tissue, whereas incomplete destruction of the glandular tissue alone produced no such effect. It was therefore decided to treat the next group of cases by destruction of the pituitary stalk alone without injection of radioactive material. Support for this method of treatment was obtained by reference to the publication of Professor D. S. Russell in which the effects of dividing the pituitary stalk in man were discussed (Russell, 1956).

Pituitary destruction by stalk section alone

Six patients have been treated by section of the pituitary stalk alone, and form Group III (Table I). Five of these were cases of carcinoma of the breast, the other was a case of carcinoma of the prostate.

The pre- and post-operative investigations were similar to those employed in Groups I and II and the same operative technique was used.

Post-operative progress

In all cases of Group III there was immediate hypothermia which was followed in 12 hours by pyrexia of 100° to 102° F. which persisted for 48 hours. Somnolence, tachycardia, and hypotension developed within 12 hours, but were abolished by intravenous hydrocortisone. A maintenance oral dose of hydrocortisone was attained in 2 weeks. Case 14 was given pre-operative intramuscular cortisone and did not develop these symptoms.

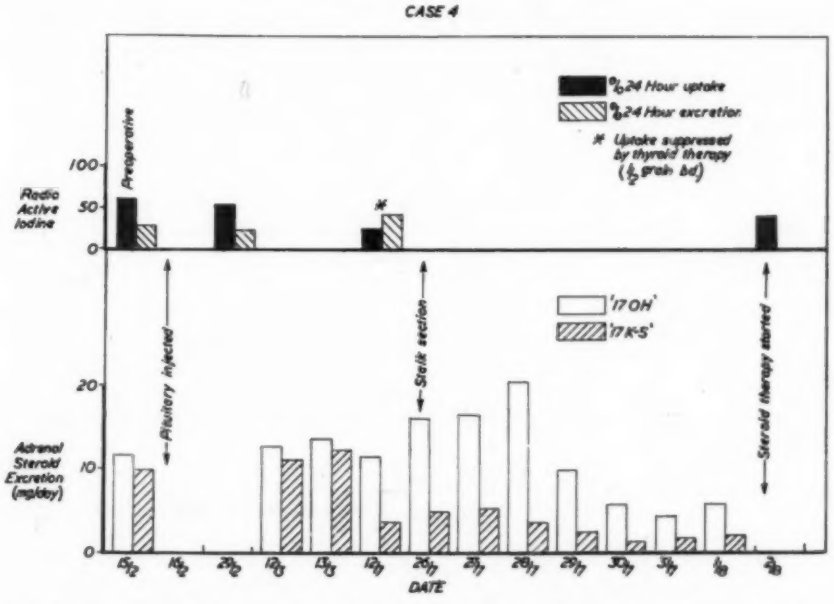


FIG. 7.—Case 4. To show post-operative fall in " 17 OH " excretion and complete suppression of radioactive iodine uptake and excretion till start of steroid therapy.

Two patients had a generalised convulsion 7 days post-operatively, the fits were controlled by intramuscular phenobarbitone.

In 4 patients there was an immediate polyuria after operation which persisted until injections of pitressin tannate were given at weekly intervals.

Headache was present in all cases but usually disappeared completely in 4–5 days.

The radioactive iodine uptake was considerably reduced in all cases post-operatively. In 1 case this occurred as early as the third post-operative day. In Case 11 the I_{131} uptake fell from a pre-operative value of 43 per cent to 7 per cent while in Case 12 the values were 31 and 3 per cent respectively. The urinary 17 OH and 17 KS rapidly decreased in all cases post-operatively. Repre-

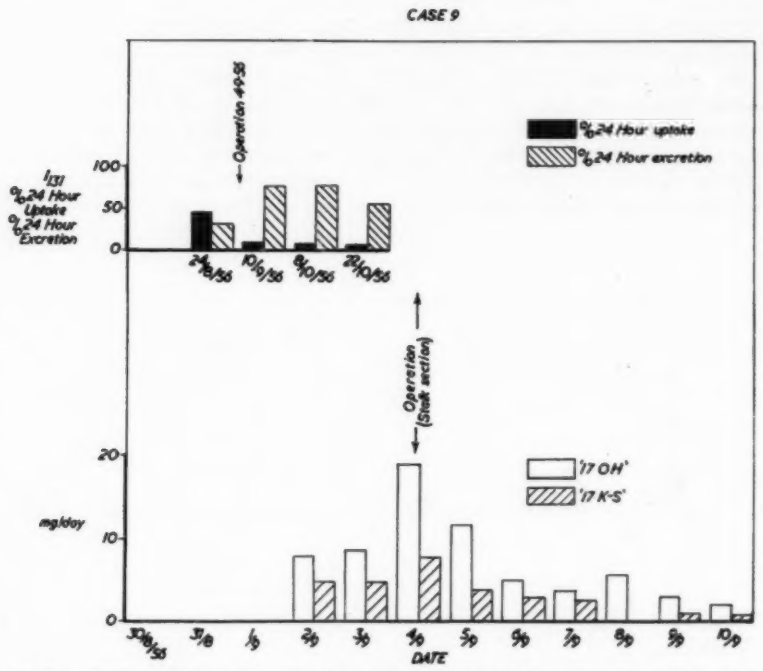


FIG. 12 (Case 9), FIG. 13 (Case 10).—To show post-operative fall in radioactive iodine uptake and steroid excretion.

sentative results of radioactive iodine and steroid excretion are shown in Fig. 12 and 13.

In Case 12, a man with skeletal metastases from prostatic carcinoma, the 24-hour urinary calcium excretions pre-operative values were 42, 52, 36, 42 mg. per 100 ml. The post-operative values were 14, 19 and 24 mg. per 100 ml. These figures suggested marked reduction in activity of the bony metastases. In this patient also there was a dramatic fall in the acid phosphatase after operation (Fig. 14).

Cases 9 and 10 had been bedridden with pathological fractures of the femora before operation. They were improved to the extent of being able to walk out of hospital after operation. In both these cases and in Case 12 there was marked relief of bone pain and subjective improvement, but despite this there were no changes in the radiological appearances of the skeleton.

Case 11 had multiple secondary deposits in the lung with a pleural effusion. She showed remarkable improvement after operation. Her pleural effusion which had required aspiration before operation did not recur; the lung deposits and the cutaneous metastases (Fig. 15) showed regression. There was no improvement in the cutaneous deposits in Case 14, 5 weeks after operation; Case 13 died 10 weeks after operation without change in her malignant disease.

All the cases in this group claimed marked improvement subjectively.

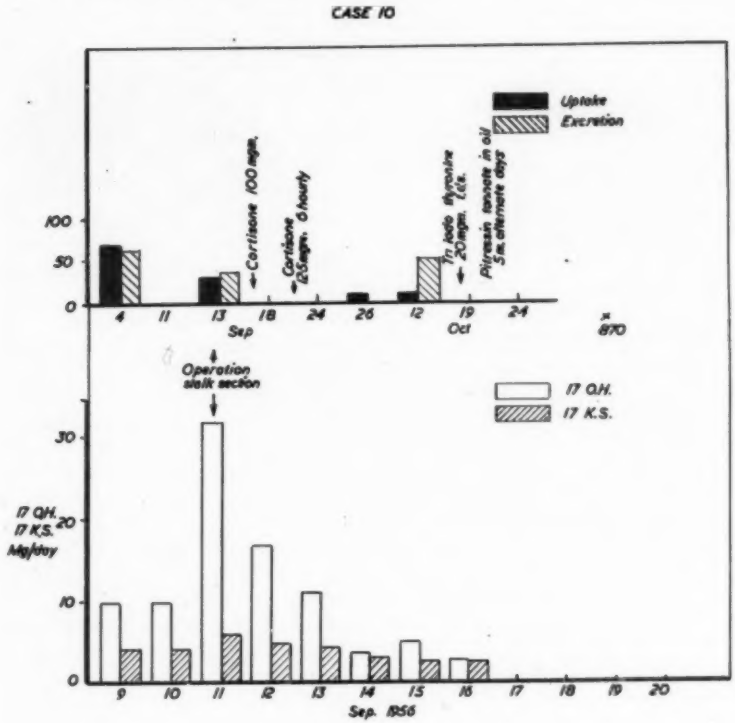


FIG. 13.

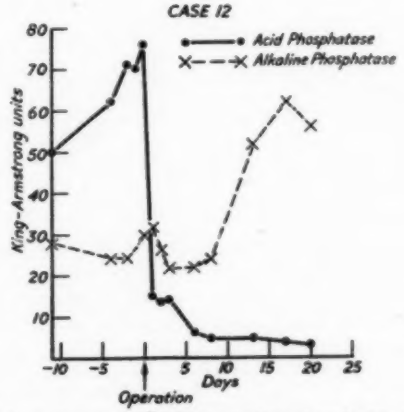


FIG. 14.—Case 12. To show changes in acid and alkaline phosphatese after stalk section in a case of carcinoma of prostate.

CONCLUSIONS AND SUMMARY

Fourteen cases of advanced malignant disease were treated by methods designed to destroy pituitary glandular tissue. In Group I, 6 cases, the pituitary gland was injected with radioactive chromic phosphate. Complete destruction of the gland was not attained in any case, nor was any improvement seen in the malignant disease for which the patients had been treated. The only case which showed any endocrine disturbance was Case 6 and at operation the pituitary stalk of this patient had been damaged.

In Group II (3 cases, 1 of which had already been treated unsuccessfully in Group I) and Group III (6 cases), the pituitary stalk was divided. Immediate and profound endocrine disturbance was evident, though the two autopsies performed on cases in Group II showed viable pituitary epithelial tissue still present. There was no apparent difference between cases treated by stalk section alone and those treated by stalk section and injection of radioactive material. Subjective improvement and relief from pain was claimed by all patients treated by stalk section and in 4 cases there was objective evidence of improvement.

It is felt that the technique of operation described for section of the pituitary stalk through a trephine hole without a major craniotomy is a relatively simple procedure ; it has proved safe in our hands even when used for seriously ill patients. It is for this reason that we decided to publish this small series at this time. It is appreciated that, though this procedure has been shown to produce severe endocrine changes suggesting suppression of pituitary glandular function, our longest follow-up is only 21 weeks after stalk section, so we do not yet know how permanent the endocrine changes may be. If pituitary function should recover at a later date due to re-growth of pituitary glandular tissue, this might be prevented by injection of radioactive material into the gland at the time of stalk section using the same operative approach.

The outstanding problem is the selection of cases of malignant disease in which regression of the tumour may be expected as a result of the severe endocrine changes produced by suppression of pituitary function.

EXPLANATION OF PLATES

FIG. 2.—Pituitary gland—Case 2. Showing partial destruction surviving tissue in left upper quadrant. H. & E. × 2·5.

FIG. 3.—Hypothalamus—Case 2. Showing nodule of tumour cells. H. & E. × 10.

FIG. 4.—As Fig. 3. H. & E. × 50.

FIG. 5.—To show operative approach.

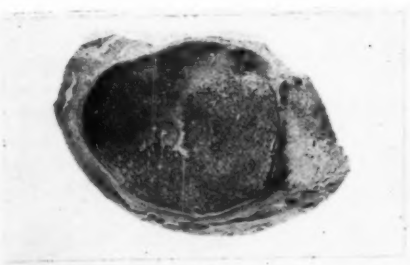
FIG. 8.—Pituitary gland—Case 7. Showing almost complete destruction. There is a small island of viable cells at the left lower border. H. &. E. × 3.

FIG. 9.—Hypothalamus—Case 7. Showing marked destruction. H. & E. × 10.

FIG. 10.—High power view of group of tumour cells just above centre of Fig. 9. H. & E.

FIG. 11.—Pituitary gland and hypothalamus—Case 8. Showing (*a*) destruction of distal part of stalk, with thrombosis of portal vessels ; partial destruction of gland—surviving tissue left lower quadrant. H. & E. × 5. (*b*) Higher power view of surviving glandular tissue. (*c*) Higher power view of pituitary stalk.

FIG. 15.—Case 11. To show regression of cutaneous metastases 2 months after stalk section.



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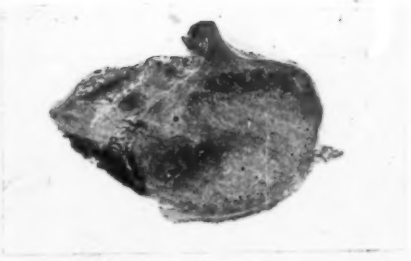

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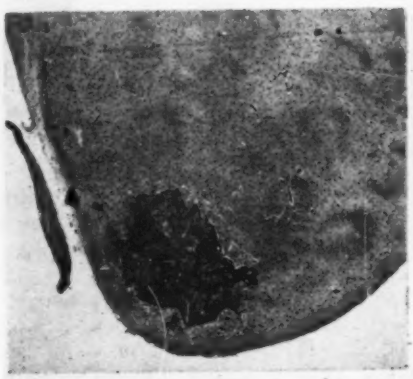

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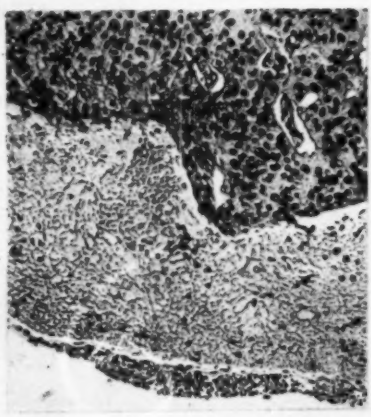

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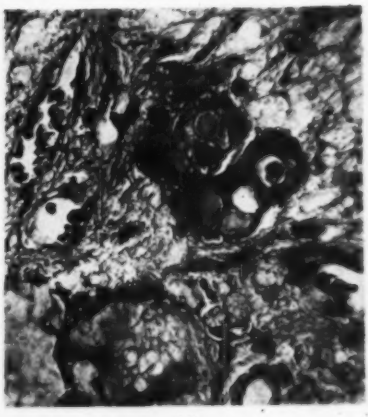

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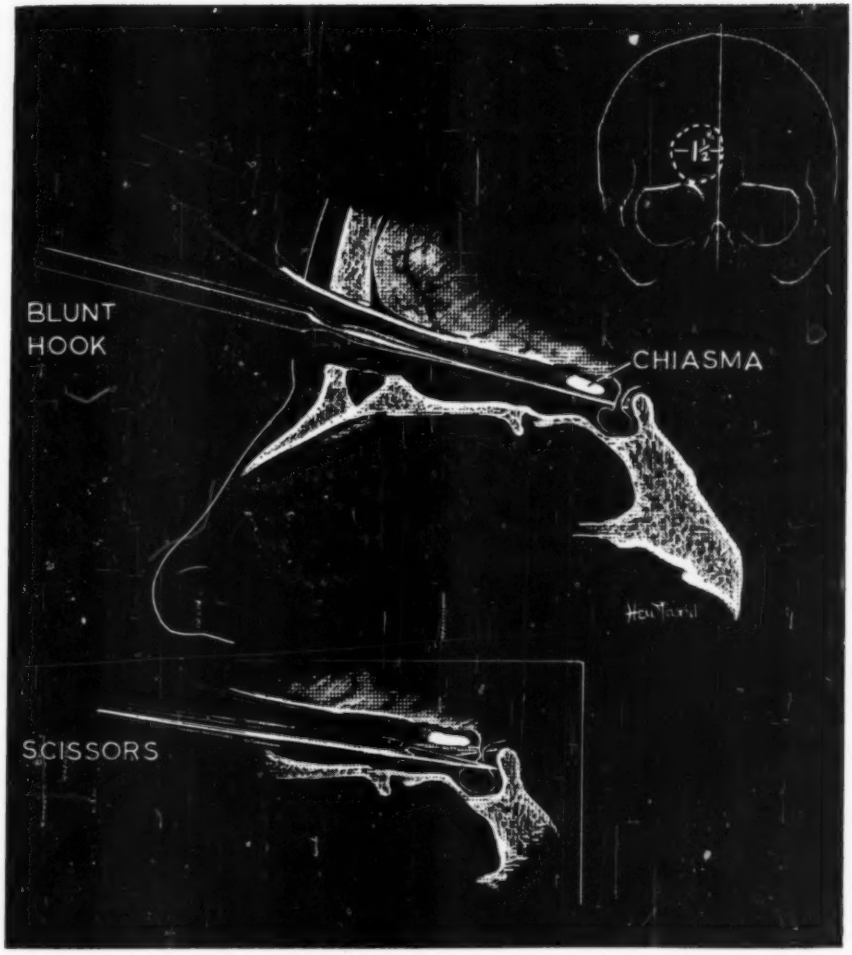

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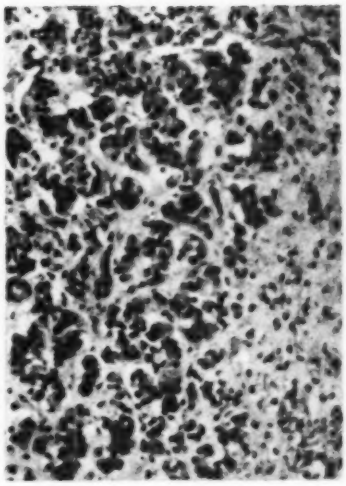

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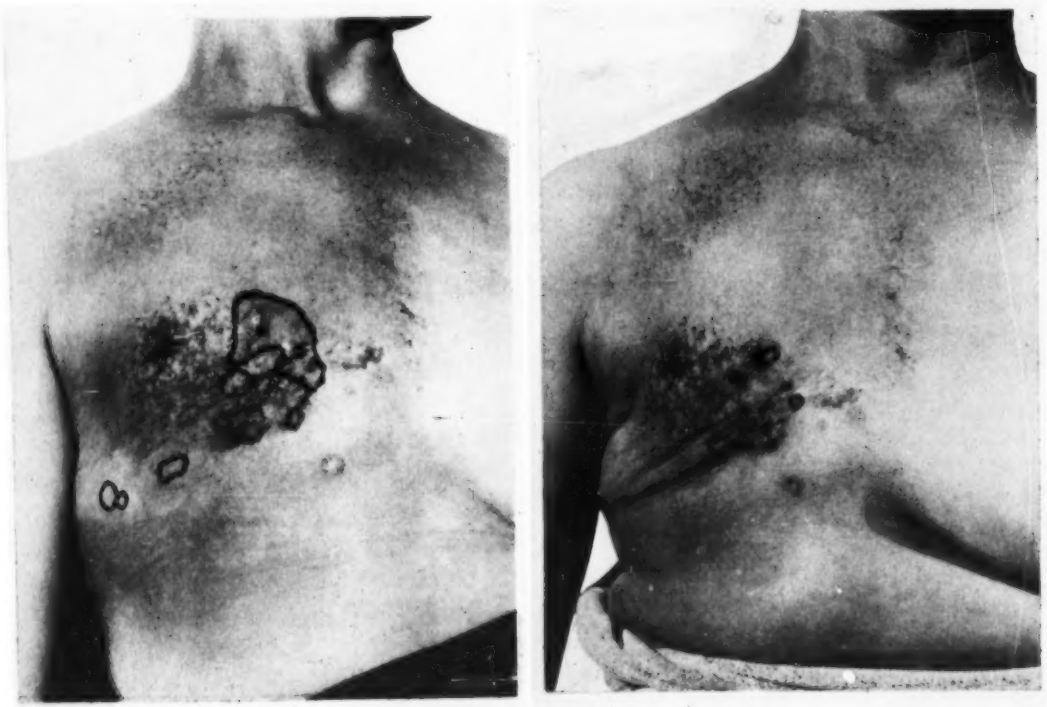

We wish to thank Dr. J. D. N. Nabarro and Dr. G. Walker who have collaborated in this work and undertaken the metabolic and endocrinological investigations which will be the subject of a later report.

Dr. A. M. Jelliffe and Mr. R. E. Ellis collaborated in the work in which radioactive chromic phosphate was used.

Our thanks are also due to Professor B. W. Windeyer, Miss M. D. Snelling, Mr. D. H. Patey and Mr. R. S. Handley for their co-operation and permission to operate on their cases.

We are grateful to Mr. T. E. Cowan for drawing the charts and to Miss E. Hewland and Mr. M. Turney for the drawings and photographs.

Part of the expenses of this investigation were met by a grant from the British Empire Cancer Campaign.

A paper based on the earlier cases in this report was read before the Society of British Neurological Surgeons in November 1956.

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ADDENDUM

Since this paper was submitted for publication Cases 9 and 12 in Group III have died, 6 and 5 months, respectively, after operation. Section of the pituitary glands in both these cases at autopsy has shown surviving pituitary glandular tissue amounting to approximately 20 per cent of normal, mainly around the periphery of the gland. There is no evidence in these cases of functional reconnection of the pituitary gland to the hypothalamus nor of re-growth of glandular tissue around the divided stalk.

CHOLEDOCHAL CYST WITH CARCINOMA OF THE INTRAHEPATIC BILE DUCTS AND PANCREATIC DUCTS

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Received for publication November 3, 1956

CHOLEDOCHAL cyst, or cystic dilatation of the common bile duct is an uncommon condition, with reports of 216 cases up to 1956 (Cautery, 1956). The clinical and pathological features have been reviewed by several authors (McWhorter, 1924; Judd and Greene, 1928; Zinninger and Cash, 1932; and Swallow, Eger and Wagner, 1943). Numerous complications of the condition have been described, cirrhosis being the most common (Gross, 1953). The development of a carcinoma is very rare, having been described in only two cases. Thus, Armanino (1946) reported a case of choledochal cyst associated with an undifferentiated carcinoma of the liver, and a carcinoma arising in the cyst wall has been described by Irwin and Morison (1944). In the present paper, a case of choledochal cyst is reported in which two distinct carcinomata developed in the intrahepatic bile ducts and one in the pancreatic ducts.

Clinical summary

The patient, a female aged 22 years, was admitted in April 1955 with a history of jaundice which had varied in intensity and had been present for seven weeks. She had had previous attacks of jaundice lasting several weeks when aged 3 and 8 years, and had been slightly jaundiced on several occasions. Physical examination showed a moderate degree of jaundice and an enlarged, smooth, firm liver. Biochemical investigations showed a raised bilirubin (6·4 mg. per 100 ml.) and alkaline phosphatase (100 KA units per 100 ml.) and a normal thymol turbidity. Urobilinogen was demonstrated in the urine on several occasions. A liver biopsy on May 4, 1955 showed a slight increase in fibrous tissue in the portal tracts, and an infiltration by lymphocytes, histiocytes and polymorphs. There was plugging of the bile canaliculi, but the larger bile ducts were empty and there was no evidence of extrahepatic obstruction. During the next five months, the patient remained jaundiced, with marked fluctuation in the bilirubin level. In November 1955 gross ascites developed. A paracentesis was performed and 9 litres of clear straw-coloured fluid were withdrawn. After the paracentesis, the liver and spleen were noted to be enlarged and of firm consistency. In December two episodes of epistaxis occurred. Her prothrombin level was found to be low but returned to normal after parenteral vitamin K therapy. By January 1956, the jaundice had disappeared. A tender swelling was noted in the abdomen to the left of the umbilicus and an enlarged lymph node was present in the left supraclavicular fossa. Biopsy of this node on January 27, 1956 showed a moderately well differentiated mucus-secreting adenocarcinoma growing in a

dense fibrous stroma. The patient developed pain in the lower part of the spine and right hip, and X-rays showed diffuse changes in the lumbar vertebrae, pelvis and both femora, the appearances suggesting widespread metastatic involvement. Both legs became swollen and numerous dilated veins were noted on the anterior abdominal wall, the appearances suggesting an obstruction to the inferior vena cava. A number of subcutaneous nodules developed around the biopsy site in the neck and on the anterior abdominal wall. The patient became slightly jaundiced again, then became comatose on March 10, 1956 and died on March 12, 1956.

Post-mortem findings

The body showed a moderate degree of jaundice and there was marked oedema of both legs. The main lesions were in the liver and extra-hepatic bile ducts.

The Ampulla of Vater and the terminal 2·5 cm. of the common bile duct were normal. The pancreatic duct opened into the common bile duct 1·5 cm. from its entry into the duodenum. There was a valve-like membrane (Fig. 1) in the common bile duct 2·5 cm. from the duodenal end, the membrane reducing the lumen to 1 mm. The common bile duct proximal to the membrane was dilated, the maximum diameter being 4 cm., and contained viscid bile. The wall of the dilated part of the duct was 0·2 cm. thick. The cystic duct was slightly dilated; the gall bladder appearing normal. There was an ulcerated carcinoma arising from the left hepatic duct near its origin, the tumour completely occluding the lumen. The tumour extended into the surrounding liver tissue forming an irregular, ill-defined mass measuring 12 × 8 × 6 cm. in the left, caudate and quadrate lobes. The left hepatic duct peripheral to the tumour was dilated forming a smooth-lined cavity 6 cm in diameter which is filled with "white bile" (Fig. 2). The right hepatic duct near its origin was narrowed, the lumen being about half its normal size. Proximal to the narrowing, the duct was dilated to a diameter of 3·0 cm and filled with viscid bile. The left lobe of the liver was almost completely replaced by tumour. The right lobe was intensely bile stained and showed a fine cirrhosis. There were numerous discrete masses of tumour in the right lobe, mostly in close relationship to the branches of the portal vein. The portal vein and its major tributaries were normal.

There were metastases in both lungs, pericardium, peritoneum, adrenal glands, thoracic and lumbar vertebrae, right femur (Fig. 3), subcutaneous tissue of anterior abdominal wall and left supraclavicular fossa, and in lymph nodes of the left supraclavicular mediastinal and para-aortic groups and in the porta hepatis. The inferior vena cava was thrombosed, the thrombus extending from 2 cm. below the entry of the renal veins to midway along the femoral veins. The brain, pituitary and thyroid glands, heart, kidneys, uterus, tubes and ovaries showed no gross abnormality. The spleen (weight 720 g.) showed thrombosis of several intrasplenic veins and a number of recent infarcts. There were a number of small metastases on the serosal surface and in the substance of this organ.

Histology

1. *Valve-like membrane in the common bile duct.*—The membrane is denuded of epithelium, the appearances suggesting that this is due to post-mortem change resulting from contact with bile. The fold consists of dense fibrous tissue in which there are several groups of smooth muscle fibres arranged circularly (Fig. 4).

Immediately distal to the membrane, there is a mass of smooth muscle fibres, the appearances suggesting a sphincter. The common bile duct below the sphincter shows a small number of smooth muscle fibres arranged longitudinally. The whole of the valve-like membrane was sectioned in order to determine whether the fold had formed at the site of entry of an anomalous pancreatic duct but no such duct could be demonstrated. Normal ganglion cells and nerve fibres are present at the duodenal end of the common bile duct.

2. *Wall of the choledochal cyst.*—The cyst wall shows no epithelial lining. The wall consists of dense fibrous tissue in which a few isolated smooth muscle fibres are present. There is no active inflammation. In sections taken from the intrapancreatic portion of the cyst, small nodules of pancreatic tissue are present within the cyst wall. A few short tubular glands are present in the wall of the cyst.

3. *Liver.*—Sections from the right lobe of the liver show an increased amount of fibrous tissue, most prominent around the medium-sized bile ducts. There is a slight increase in the fibrous tissue of the portal tracts and in some areas the fibrous tissue extends to surround the liver lobules (Fig. 5). The most prominent feature in the portal tracts is a well marked hyperplasia of the bile ducts (Fig. 6). There is a widespread, irregular infiltration of the portal tracts by an adenocarcinoma consisting of cuboidal cells forming irregular acini and growing in a fibrous stroma. While in most areas the distinction between bile duct hyperplasia and carcinomatous invasion is readily made, in other areas it is impossible and in some there appears to be a continuous gradation from the hyperplastic bile ducts to carcinoma (Fig. 7). Sections from the left lobe show only a few small remaining groups of liver cells. The remainder consists of carcinoma growing in a dense fibrous stroma. A number of small and large branches of the portal vein are invaded by tumour, and some are occupied by thrombus containing tumour.

4. *Left hepatic duct.*—The sections taken from the origin of the duct show an adenocarcinoma arising from the duct (Fig. 8). The tumour consists of irregular acini lined by cuboidal cells with large hyperchromatic nuclei. Many of the acini, and a few of the cells contain mucin. The dilated left hepatic duct is lined by a flattened pavement type of epithelium with a slight lymphocytic exudate in the submucosa. The duct is surrounded by tumour which has invaded the muscle coat and submucosa in some areas.

5 *Right hepatic duct.*—Sections taken from the narrowed part of the duct show an adenocarcinoma arising from the duct, with extensive invasion of the surrounding tissues. The appearances are similar to those of the tumour arising from the left hepatic duct.

6. *Left hepatic vein.*—There is an organized thrombus in which tumour is present, the thrombus occupying about one-half of the lumen.

7. *Pancreas.*—The body of the pancreas shows no abnormality. In the section showing the valve-like membrane in the common bile duct, there is a small area of pancreas, measuring 1·5 × 1·0 cm., showing a chronic pancreatitis with marked interlobular and some intralobular fibrosis. In addition, there is a papillary carcinoma arising from a medium-sized pancreatic duct (Fig. 9) and extending along the smaller ducts where the appearances are those of carcinoma *in situ*. There is also invasion of the surrounding tissue. The tumour consists of tall columnar mucus-secreting cells of the goblet cell type, with large oval, rather

vesicular nuclei and prominent nucleoli. The histological appearances of this tumour are quite distinct from those of the tumours arising from the bile ducts.

8. *Other organs.*—There are metastases in the lungs, bones, adrenal glands, pericardium and peritoneum, lymph nodes, left ovary and spleen. All the metastases show a similar structure to the carcinoma arising from the bile ducts. The kidneys show hyaline droplet change of the neck of the proximal tubules, and numerous bile-stained tubular casts. The thyroid and pituitary glands, heart muscle and uterus show no abnormality.

DISCUSSION

The case presents the typical features of a choledochal cyst. The aetiology of this condition has given rise to much speculation, no less that fifteen theories being quoted by Swallow, Eger and Wagner (1943). A simpler approach is that of Ladd and Gross (1951) who suggest that two factors are necessary, a congenital weakness of the wall of the common bile duct, together with some factor producing obstruction. The weakness of the duct wall has been attributed to the presence of pancreatic tissue in the wall (Budde, 1920), a feature noted in the present case. However such tissue is not constantly present (McWhorter, 1924) and may be found frequently in the normal common bile duct (Burden, 1925 ; Cross, 1956). A number of factors have been described as producing obstruction to the common bile duct, these including an abnormal course or stenosis of the intraduodenal part of the duct (Rostowzew, 1902 ; Edgeworth, 1895), pressure of the pregnant uterus (Goldammer, 1907), neuromuscular inco-ordination as in megacolon (Bohmansson, 1924), and a valve-like membrane in the duct (Rostowzew, 1902 ; Waller, 1917). In the present case the course and lumen of the intraduodenal part of the duct were normal, and there were normal ganglion cells and nerve fibres in the duct wall. The patient had never been pregnant. The obstruction in this case appears to be due to a valve-like fold in the common bile duct, a feature which was present in one-third of the series reviewed by McWhorter (1924). In many of the recent cases the diagnosis of choledochal cyst has been made during life and operative treatment undertaken, and in consequence the nature of the obstruction has not been determined. The structure of the valve-like membrane has received little attention, no account being given in any of the case reports. In the present case, the fold consists of mature fibrous tissue in which a smooth muscle sphincter is present. The origin of this valve-like fold presents some difficulty. No satisfactory embryological basis can be suggested and the structure does not indicate an inflammatory origin. The possibility that it occurred at the site of entry of an anomalous pancreatic duct (Cross, 1956) was considered, but no such duct could be demonstrated in serial sections through the fold. The normal common bile duct shows a number of small valvules in the region immediately proximal to the entry of the pancreatic duct (Sterling, 1949). These valvules consist of muscle and fibrous tissue and are normally quite small. Their function is the prevention of regurgitation of intestinal contents into the duct (Schwegler and Boyden 1937). The valve-like fold in the present case is thought to represent an unusually prominent valvule, a conclusion supported by the site and structure of the fold. The distance of the fold from the opening into the duodenum is accounted for by the pancreatic duct joining the common bile duct much earlier than normal (Sterling, 1956).

A choledochal cyst may be followed by numerous complications, of which biliary cirrhosis is the most common (Gross, 1953). In the forty-eight cases reviewed by McWhorter (1924), no less than fourteen of the thirty in which adequate descriptions of the liver were given showed cirrhosis. The cirrhosis may result from the obstruction to the common bile duct, or may follow the cholangitis which occasionally occurs. The absence of cirrhosis in many cases may be attributed to the absence or intermittency of the obstruction. Thus, jaundice is absent in 20–30 per cent of cases (Dickinson and Spencer, 1952) and the intermittency of the clinical picture has been emphasized by Gross (1953). In a case of choledochal cyst reported by Gross (1933), a liver biopsy showed a marked biliary cirrhosis, while a second biopsy, taken six weeks after the relief of the obstruction, showed a marked reduction in fibrous tissue, and a regeneration of the hepatic parenchyma. Similarly Cameron and Oakley (1932) have shown that the changes produced in experimental animals by tying the common bile duct undergo regression when the obstruction is relieved, a finding confirmed by Gillman, Gilbert and Spence (1954). In the present case, while there is a marked hyperplasia of the bile ducts, there is only a slight increase in fibrous tissue in the liver and the lobular architecture is well preserved.

The most unusual feature of the present case is the development of a bile duct carcinoma. Sections from the right and left hepatic ducts show a carcinoma arising from each duct, with normal mucosa intervening. There is widespread invasion of the portal tracts by tumour, the appearances being consistent with either multifocal origin or intrahapatic spread. However, while in most areas it is possible to distinguish between bile duct hyperplasia and carcinomatous invasion, in some sections this distinction is not possible and in others a continuous gradation from hyperplastic bile ducts to carcinoma can be seen. The appearances, then, are of a bile duct carcinoma of multifocal origin. A unique feature is the occurrence of a further carcinoma arising from the ducts of a small pancreatic lobule. While the point of entry of the duct draining this lobule could not be demonstrated, it is probable that the duct entered the common bile duct proximal to the valve-like fold, so that the lobule would be subjected to the same abnormal environment as the liver. The carcinoma of the bile ducts developed in a liver in which extensive bile duct hyperplasia had resulted from the obstruction to the common bile duct. The process of bile duct hyperplasia following complete and partial obstruction to the common bile duct has been extensively studied in several experimental animals (Cameron and Oakley, 1932 ; Gillman, Gilbert and Spence, 1954). While marked degrees of hyperplasia were seen after prolonged obstruction, no tumours developed. In the experimental animals, a single period of obstruction was used, but in the present case at least three major and several minor episodes of obstruction occurred. Similar repeated attacks of obstruction were also a feature of the previous case of carcinoma of the liver in association with a choledochal cyst (Armanino, 1946). Thus, while a single episode of obstruction only results in bile duct hyperplasia, it seems possible that a repeated stimulus may produce malignant change in the hyperplastic ducts. A similar situation exists in the development of a carcinoma in association with cirrhosis, where a single short-lived episode of liver damage is not likely to be followed by carcinoma. It is the progressive destruction of liver cells followed by progressive hyperplasia that may lead to the development of carcinoma (Stewart, 1931).

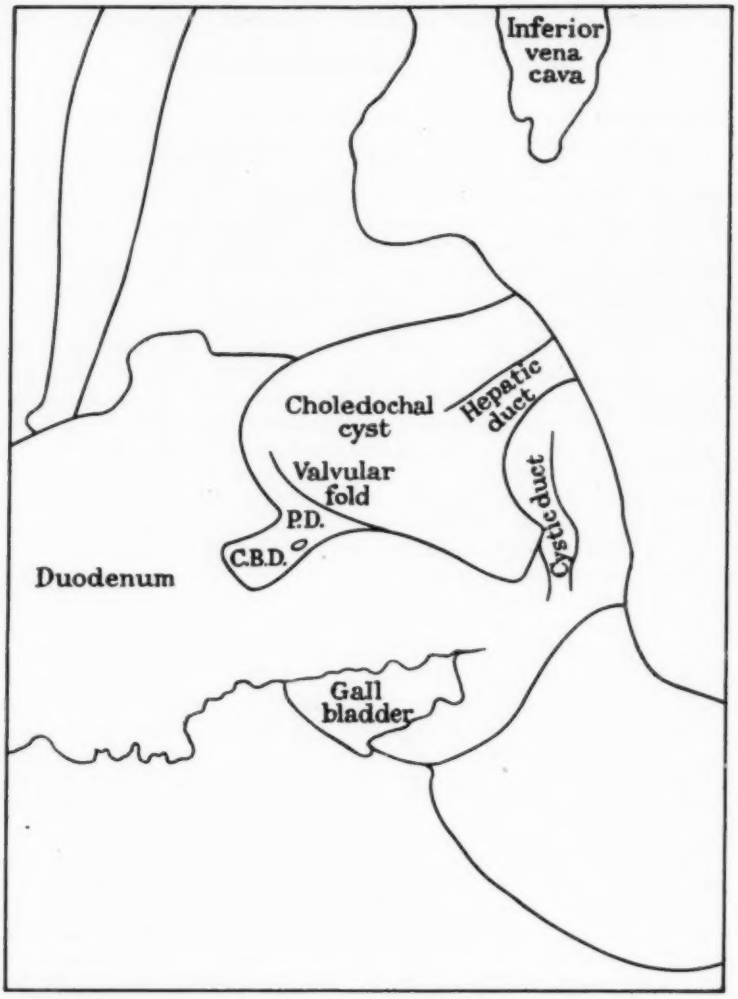
In view of the close structural similarity between the bile acids and methyl cholanthrene and the *in vitro* production of methyl cholanthrene from bile (Wieland and Dane, 1933; Fieser and Newman, 1935), the role of the bile acids in the genesis of the tumour must be considered. There is no experimental evidence that cholic acid is carcinogenic, but Gillman, Gilbert and Spence (1954) suggest that in the presence of obstruction abnormal metabolites may be formed. A number of pathogenic bacteria are capable of metabolizing bile acids *in vitro*, with the production of several metabolites (Fukui, 1937; Mori, 1939; Hoehn, Schmidt and Hughes, 1944; Hughes and Schmidt, 1942; Schmidt and Hughes, 1942; and Schmidt, Hughes, Green and Cooper, 1942), and thus infection secondary to the obstruction may result in the bile ducts being exposed to abnormal substances. However, none of these metabolites have been shown to be carcinogenic so far. Most attempts to produce bile duct carcinoma in experimental animals by the injection of bile and liver extracts from patients with carcinoma have been unsuccesssful, but Fortner and Norris (1955) in a small-scale experiment produced a bile duct carcinoma in guïnea-pigs by the injection of bile from cases of human bile duct carcinoma.

Gillman, Gilbert and Spence (1954) have demonstrated extensive bile duct hyperplasia in animals fed on a diet deficient in vitamin A. In the presence of prolonged obstructive jaundice, a deficiency of fat soluble vitamins may occur. In the present case, vitamin K deficiency resulted in a hypoprothrombinaemia, which was corrected by parenteral therapy. While there was no clinical evidence of hypovitaminosis A, it is probable that some deficiency existed and that this contributed to the bile duct hyperplasia. In experimental animals, bile duct carcinoma develops as a consequence of hyperplasia (Gillman, Gilbert and Spence, 1954) and while this is not usually seen in association with human bile duct carcinoma, the findings in the present case indicate that this sequence of events took place. The bile duct hyperplasia in this case may be attributed to the repeated obstruction, abnormal bile acid metabolites, and hypovitaminosis A. While there is little evidence that any of these factors acting separately is carcinogenic, it seems probable that the unique combination present in this case was responsible for the development of the carcinomata.

The hepatic tumours present in this case are clearly of bile duct origin. The case presents considerably similarity to the first case of carcinoma arising as a complication of a choledochal cyst (Armanino, 1946), though in that instance the tumour was an anaplastic carcinoma. It was, however, considered to be of bile duct origin and the presence of a fibrous tissue stroma would support such an origin (Stewart, 1931). The tumour reported by Irwin and Morison (1944) presents a number of different features. It occurred in a man aged 30 and arose from the cyst wall. The cyst contained a number of gall stones, this being an uncommon occurrence in choledochal cyst (Keeley, 1948). The tumour was a squamous cell carcinoma and was probably similar in origin to those sometimes seen in the gall bladder in association with calculi, though the age and sex of the patient suggest that other factors may also have been involved.

SUMMARY

(1) A case of choledochal cyst associated with a carcinoma of the bile ducts of multifocal origin and a carcinoma of a small pancreatic duct is reported.



C.B.D. Common bile duct P.D. Pancreatic duct

EXPLANATION OF PLATES.

FIG. 1.—Liver, bile ducts and duodenum showing opened common bile duct. The terminal part of the duct is normal and shows the entry of the pancreatic duct. There is a valve-like fold in the common bile duct with gross dilatation proximal to it (slightly reduced.)

FIG. 2.—Cut surface of the liver showing the tumour with dilatation of the intrahepatic bile ducts. There are several discrete tumour masses. There are thrombi in branches of the portal vein. (Slightly reduced.)

FIG. 3.—Section of right femur showing numerous secondary deposits. × ¼.

FIG. 4.—Section of the valve-like fold showing the presence of smooth muscle fibres. Phloxin-tantrazine. × 20.

FIG. 5.—Section of livers howing increase in fibrous tissue around the larger bile ducts and the liver lobules. H. & E. × 22.

FIG. 6.—Portal tract showing bile duct hyperplasia. H. & E. × 125.

FIG. 7.—Portal tract showing bile duct hyperplasia and carcinoma. There appears to be a gradation from hyperplasia to carcinoma. H. & E. × 145.

FIG. 8.—Left hepatic duct showing the origin of the bile duct carcinoma. H. & E. × 38.

FIG. 9.—Small pancreatic duct showing the origin of carcinomafrom the epithelium. H. & E. ×95.

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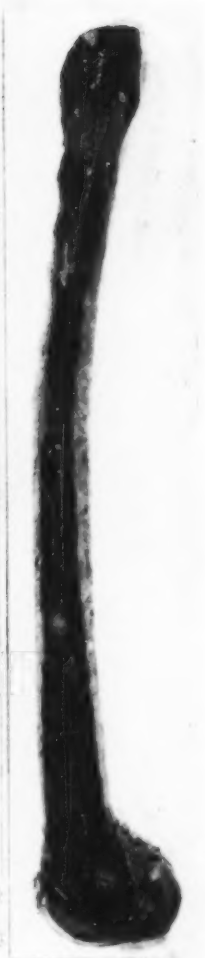

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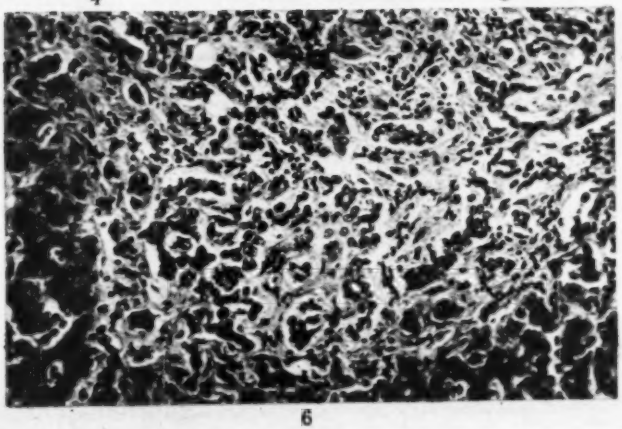

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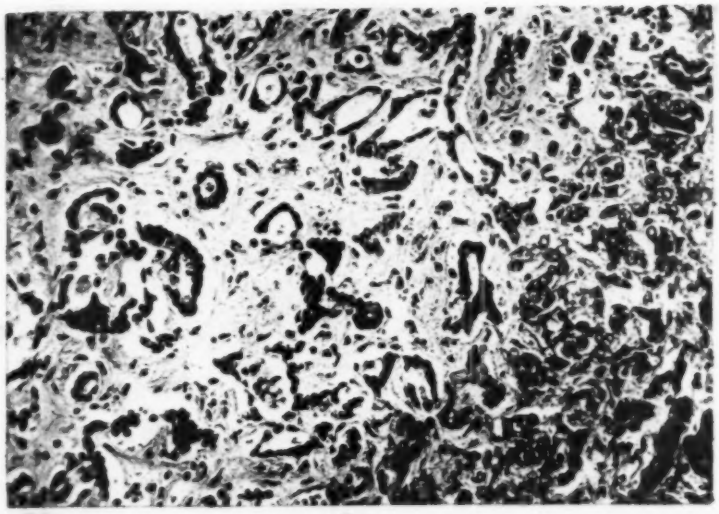

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(2) The choledochal cyst resulted from the presence of a valve-like fold in the common bile duct. The structure of the fold is described and its origin discussed.

(3) The factors responsible for the development of the carcinomata are discussed.

I wish to express my thanks to Professor T. Crawford for his advice and encouragement in the preparation of this paper and to Dr. A. Hunter for permission to use the clinical notes.

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RETINAL ANLAGE TUMOUR OF THE MAXILLA

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Received for publication January 24, 1957

THE lesions now generally described as tumours of retinal anlage occur in infants under the age of 12 months and are usually situated in the maxilla.

The first case of retinal anlage tumour to be designated as such was reported by Halpert and Patzer (1947) and 6 more cases have since been recorded, but it has also become apparent that the melanotic epithelial odontome reported years before by Mummery and Pitts (1926), together with the pigmented ameloblastomas or adamantinomas subsequently recorded by other authors, should be considered in the same context. Moreover, there are cases of tumours of the jaws in infants described as melanocarcinomas or melanomas which would appear to warrant consideration. In all, there are 20 cases in the literature which have been reported under one or other of the designations already mentioned and which seem to fall into a single group. These cases comprise 14 tumours of the maxilla, 4 of the mandible, 1 of the skull and 1 of the epididymis, and an additional case of a maxillary tumour is reported here. The age, sex, site of tumour and designation of these cases are summarised in Table I.

CASE REPORT

The patient was a female infant aged 6 months, brought to hospital because of a swelling in the left upper incisor region. It is not known how long the swelling had been present. The tumour was excised, but unfortunately no further clinical details are available.

Microscopically, the tumour consists of groups of cells in a plentiful connective tissue stroma. Two main types of cell are present in these groups ; small cells which are non-pigmented and larger cells which contain pigment, generally in abundance. The smaller cells, which are round and contain well-stained nuclei but have little cytoplasm, resemble neuroblasts and are arranged in groups or follicles. In some groups there is very little intercellular material (Fig. 1) but in others there is a fibrillar matrix between the cells, which resembles glia (Fig. 2 and 3). Among these small round cells is seen an occasional larger cell with a triangular cell body and evidence of a cytoplasmic process at one pole, and occasionally there may be seen transitions to nerve-like structures (Fig. 2 and 4).

The larger cells are cuboidal or rather flattened and contain masses of dark brown iron-free pigment. This is often so copious that the cell outlines and nuclei are obscured, but where the pigment is less plentiful, or towards the periphery of the larger clumps, it can be seen that it takes the form of minute, rod-shaped particles. These pigment-containing cells line cleft-like spaces which may be empty or may contain groups of the smaller cells already described (Fig. 5 and 6).

TABLE I.

	Author	Sex	Age in months	Site	Designation
1	Mummery and Pitts (1926)	F.	5½	Maxilla	Melanotic epithelial odontome.
2	Dudits and Szabó (1935)	,,	1½	,,	Congenital melanocarcinoma.
3	Soderberg and Padgett (1941)	,,	1½	,,	Melanoma.
4	,, ,, ,, ,,	,,	1	,,	,,
5	Halpert and Patzer (1947)	,,	6	,,	Retinal anlage tumour.
6	Field, Ackerman and Kannerstein (1950)	,,	1½	,,	Melanotic tumour of maxilla.
*7	Bernier	,,	1½	,,	Pigmented ameloblastoma.
*8	,,	,,	12	,,	,, ,,
9	Martin and Foote (1951)	,,	4	,,	Retinal anlage tumour.
10	Shafer and Frissell (1953)	,,	4	,,	,, ,, ,,
11	Hünerwald (1953)	,,	6	,,	Congenital melanotic tumour.
12	Notter and Söderberg (1953)	M.	2	,,	Melanocarcinoma.
13	Macdonald and White (1954)	,,	2	,,	Pigmented congenital epulis of neuro-epithelial origin.
14	Caldwell, Ernst and Thompson (1955)	,,	3½	,,	Retinal anlage tumour.
15	Krompecher (1918)		2	Mandible	Congenital melanocarcinoma.
16	Wass (1948)	F.	5	,,	Melanotic adamantinoma.
17	Battle, Hovell and Spencer (1952)	M.	1½	,,	Pigmented adamantinoma.
*18	Bernier	F.	3	,,	Pigmented ameloblastoma.
19	Clarke and Parson (1951)	M.	9	Skull	Retinal anlage tumour.
20	Eaton and Ferguson (1956)	M.	5	Epididymis	Retinoblastic teratoma.

* Cited by Caldwell, Ernst and Thompson (1955).

DISCUSSION

There seems good reason to believe that the cases of pigmented tumours of the jaws and elsewhere listed in Table I are all examples of the same entity, since the clinical and pathological features are so similar.

The age and sex incidence are very striking, for in all cases the patient has been an infant aged 12 months or less, and females greatly preponderate. All of the tumours have so far proved benign, and though the follow-up period in some cases has been only a matter of months there are several which have been observed over longer periods, up to 7 years. Histologically, also, these cases appear to be very similar, so far as may be judged from descriptions and photomicrographs.

With regard to the nature of the lesions, there have been three main theories. Krompecher (1918), Dudits and Szabó (1935) and Soderberg and Padgett (1941) considered their cases to be melanomas, deriving from odontogenic epithelium or from epithelial cells enclaved in the course of fusion of the maxillary processes.

Mummery and Pitts (1926), however, were of the opinion that the tumour in their case derived from odontogenic epithelium and that it should be considered as a type of epithelial odontome, that is to say, as an adamantinoma, and this view was also adopted by such later authors who reported their cases under the titles of pigmented ameloblastoma or adamantinoma. But the only cases in

which detailed attention has been paid to the relationship of the tumour to the dental tissues are those of Krompecher (1918), Mummery and Pitts (1926) and Dudits and Szabó (1935). In these cases developing teeth were found in proximity to the tumour tissue, but there is no clear evidence to show that the neoplasm had in fact originated from dental epithelium. On the contrary, the developing teeth could well have been encroached upon and displaced by the growing tumour.

Halpert and Patzer (1947) were the first to suggest the retinal nature of these growths, pointing out that the cleft-like spaces lined by pigmented cells sometimes contain infoldings which resemble the ciliary processes of the eye, and that the smaller cells resemble those seen in the nuclear layers of the retina or the cells in neuroblastomas. Later authors have all agreed in general with the neural concept of histogenesis, and follow Halpert and Patzer's view that retinal tissue, misplaced in the course of development, is the origin of these growths. The small round cells seen in the tumours are considered to be neuroblasts, with differentiation into glial cells, spongioblasts and primitive retina. The fact that the pigment granules in the cuboidal cells show the characteristic bacillary morphology of retinal pigment is pointed out by Macdonald and White (1954), who also note the similarity between the tumour and the embryonic eye at an early stage when the retina is still composed of neuroblasts, and later when the optic cup has formed.

The view that such tumours are of neuro-epithelial derivation seems, therefore, to be inescapable, and it seems reasonable to attribute their origin to primitive retinal elements which have become displaced in the course of development. All of the tumours, except one, have occurred in sites where such displaced elements might readily be found; the exception is the tumour of the epididymis, which the authors consider as possibly representing a one-sided development of a teratoma arising from a totipotent cell rest.

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EXPLANATION OF PLATE

Fig. 1.—Follicles of small round cells with little intercellular material are present in a connective tissue matrix. × 55.

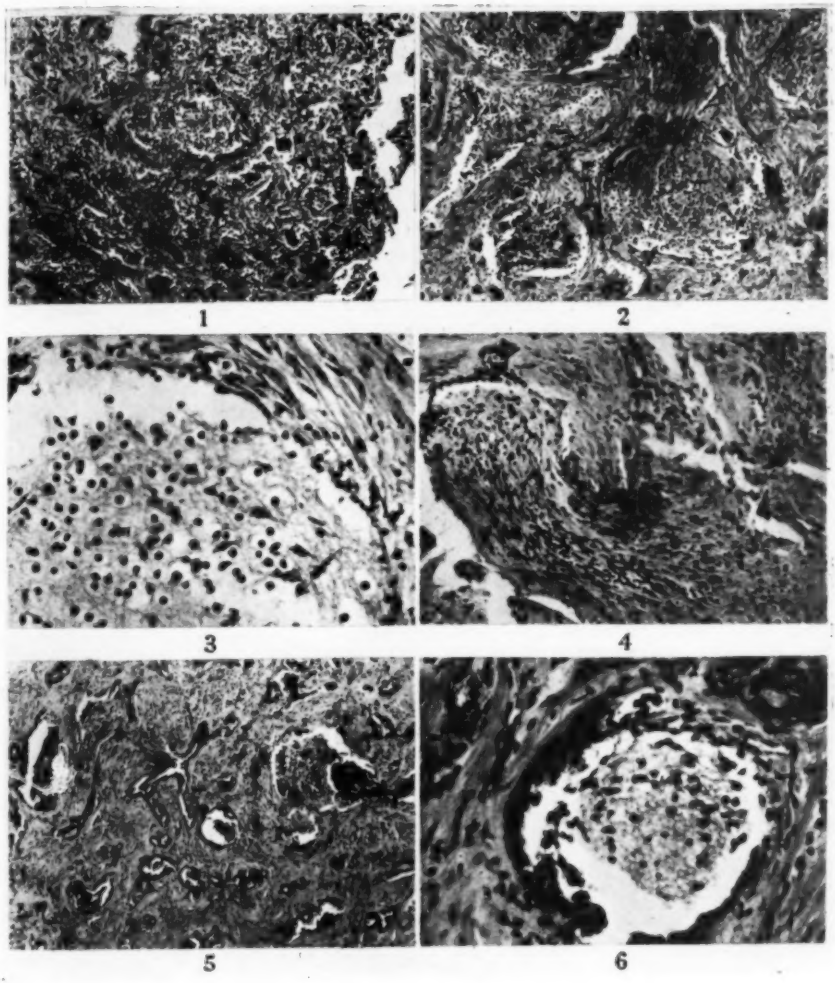
Fig. 2.—The cellular aggregations show a fibrillar intercellular matrix resembling glia, and differentiation towards nerve-like structures can be seen. × 55.

Fig. 3.—Detail from a field similar to that shown in Fig. 2, to show the glia-like nature of the intercellular fibres and the presence of occasional larger cells with polar processes. × 225.

Fig. 4.—Differentiation of nerve fibres from the neuroblastic cells. × 225.

Fig. 5.—Cleft-like spaces lined by pigmented cells. × 55.

Fig. 6.—A space lined by pigmented cells and containing neuroblastic cells and glia-like fibres. × 225.



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ALVEOLAR METAPLASIA: ITS RELATIONSHIP TO PULMONARY FIBROSIS IN INDUSTRY AND THE DEVELOPMENT OF LUNG CANCER

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Received for publication November 27, 1956

MANY factors are probably responsible for the rising incidence of lung cancer and one may be the increasing exposure of the population to industrial dusts and fumes. Peripheral lung scars have been suggested as a site of origin (Womack and Graham, 1941; Prior, 1953; Raeburn and Spencer, 1953; Spencer and Raeburn, 1954, 1956; King, 1954). Areas of lung fibrosis often contain dust pigment and may be associated with epithelial metaplasia of adjacent air spaces. Few studies, however, have been made of epithelial changes in relation to fibrosis in lungs of industrial workers. If the dust is carcinogenic then one might expect an increased frequency of metaplasia or atypical epithelial changes possibly related to the development of carcinoma, occurring at these sites.

This paper reports an analysis of epithelial changes in distal air spaces related to areas of fibrosis in lungs from workers with and without carcinoma in industries with varying incidence of lung cancer. The fibrosis may be local or general, local as in a tuberculous scar, general as in asbestosis. "Alveolar metaplasia" will be used to denote change in the epithelium of distal respiratory air spaces to distinguish it from bronchial metaplasia which usually refers to non-respiratory bronchi. Many of the distal air spaces are alveoli, but in the absence of serial sections in every case, exact identification is impossible. The spaces therefore include alveoli, alveolar ducts and respiratory bronchioles. The relationship of alveolar metaplasia to age, occupation, presence or absence of carcinoma, chronic venous congestion, oedema and pyogenic infection will be ascertained. Sex differences were not investigated as the majority of cases were men. This investigation is not concerned with metaplasia proximal to respiratory bronchioles.

It is widely known that simple benign cuboidal metaplasia of alveoli (alveolar metaplasia) is often seen in areas of oedema, congestion, collapse, bacterial and viral infections, tuberculosis, and any non-industrial lung fibrosis (Geever, Neuberger and Davies, 1943). This appearance is illustrated in Fig. 1 and will be referred to as "typical metaplasia". The section is taken from a housewife with no industrial exposure whose lungs at autopsy showed diffuse fibrosis following repeated episodes of infection. The respiratory air passages are lined by a single layer of regular cuboidal epithelium. The site of origin of these cells, alveolar or bronchial is beyond the scope of this investigation. Any deviation from this appearance, such as irregularity of size, staining or mitotic activity will be considered as "atypical alveolar metaplasia".

MATERIAL AND METHODS

Four main groups of industries were studied, selected according to their cancer incidence.

Group I.—Unaffected cancer incidence, coal workers' pneumoconiosis (James, 1955).

Group II.—Unaffected, but with some experimental evidence of carcinogenesis, berylliosis (Schepers, *et al.*, 1957; Vorwald, personal communication).

Group III.—Possibly increased cancer incidence, e.g., tar workers (Hueper, 1942, 1956; Wynder and Graham, 1951).

Group IV.—Increased cancer incidence, e.g. asbestos workers (Doll, 1955).

Where the whole lung was available for study, multiple blocks were examined, some by serial section. These were taken from any area of fibrosis of occupational or non-occupational origin. From some of the cases histological sections only were available so that examination was not so complete. The sections were usually stained by haematoxylin and eosin.

No conclusion as to the incidence of lung cancer in these industries is possible from this material as it is partially selected.

Group I : Industries with unaffected cancer incidence

Coal workers' pneumoconiosis was taken as an example of this group. Unlimited material was available. Histological sections from lungs of one hundred consecutive autopsies were examined and in this series there were no cases of carcinoma. Sections from thirty additional cases were selected, being consecutive cases with carcinoma. Two types of lesions are described in this disease—simple and massive (Gough, 1947; Heppleston, 1951). Simple pneumoconiosis consists of small collections of coal dust with minimal reticulin fibrosis and associated focal emphysema. Massive or progressive massive fibrosis, P.M.F., is thought to be tuberculosis modified by coal dust with a great increase of connective tissue. In an autopsy series James (1955) found lung cancer in 3·3 per cent of South Wales coal miners, compared with 5·4 per cent in a control series of non-miners. Multiple sections were available from each lung in every case so that many hundreds of scars of both types were examined, some in serial section.

Group II : Industries with unaffected cancer incidence but with exposure to agents thought to be carcinogenic to animals (Table I)

The only disease included in this group is *Berylliosis*. It may occur in both an acute and a chronic form. Cases of both types were examined but the results refer only to the chronic variety. Fifty-two cases (Table I) were studied, all without carcinoma. In the majority of instances the lungs were not available so that examination was confined to existing histological sections. This was of no real disadvantage as all the sections showed the characteristic lesions, but meant that non-occupational scars were not often examined. This disease gives rise to widespread interstitial fibrosis associated with focal "sarcoid"-like granulomata (Hardy and Tabershaw, 1946; Dutra, 1948; Saranac Symposium, 1950).

TABLE I.—*Chronic Berylliosis* (52)

Occupation	Total
Fluorescent lamp industry	18
Living near Be plant " neighbourhood cases "	10
Beryllium alloy workers	9
Beryllium factory worker (type of work unspecified)	6
Laboratory workers	4
No details of exposure	2
Cathode tube factory	1
Ceramic factory	1
Glass factory	1

There is some experimental evidence that prolonged beryllium inhalation in rats produces lung cancer (Schepers *et al.*, 1957; Vorwald, personal communication). Dutra, Largent and Roth (1951) on exposing rabbits to beryllium oxide inhalation produced metastasising osteosarcoma as did Vorwald (1950) by intravenous injection. There is no evidence at present to incriminate beryllium as being carcinogenic to man. It may be that diseased patients have not lived for a sufficient length of time to develop carcinoma. If the experimental production of lung cancer is confirmed the time factor may be of real importance in the development of human cases.

Group III : Industries with possibly increased cancer incidence

Oil and tar (Table II).—The four men in this group had carcinoma of main bronchi. The whole lungs were available for study so that sections from both local and general areas of fibrosis were examined, some in series. Hueper (1942), in reviewing the literature, is of the opinion that such workers may well have an increased incidence of lung cancer. Tobacco " tars " have not been considered in the present study though they may be a most important member of this group.

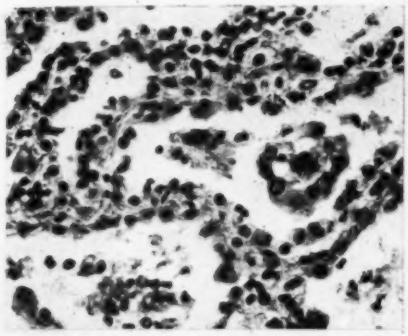
TABLE II.—*Tar and Oil Workers* (4—*All with Carcinoma*)

Occupation	Total	Carcinoma
Oil burner mechanic	1	Squamous
Oil stoker	1	"
Machine shop oiler	1	Epidermoid
Roof contractor (tar)	1	"

Haematite miners.—Histological sections from ten unselected cases, two with carcinoma, were examined. Bonser, Faulds and Stewart (1955) found an increased

EXPLANATION OF PLATES

FIG. 1.—Typical alveolar metaplasia in a non industrial lung. H. & E. × 265.
FIG. 3.—Typical alveolar metaplasia in a coal miner. H. & E. × 265.
FIG. 4.—Typical alveolar metaplasia in a beryllium lung. H. & E. × 280.
FIG. 5.—Endothelioma of the pleura invading underlying lung in a haematite miner. H. & E. × 195.
FIG. 6.—Slightly atypical alveolar metaplasia in an asbestos lung. H. & E. × 210.
FIG. 7.—Slightly atypical alveolar metaplasia in an abestos lung. H. & E. × 215.
FIG. 8.—Alveolar cell carcinoma in an asbestos worker. H. & E. × 255.
FIG. 9.—Atypical alveolar metaplasia in a nickel lung. H. & E. × 495.
FIG. 10.—Alveolar cell carcinoma in a nickel worker. H. & E. × 125.



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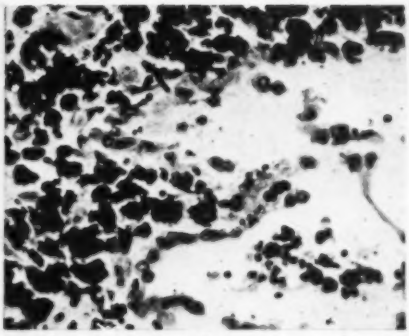

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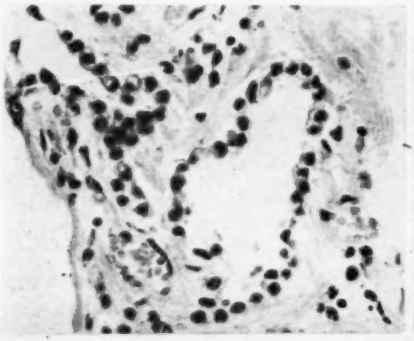

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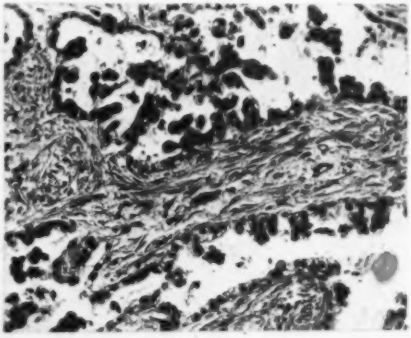

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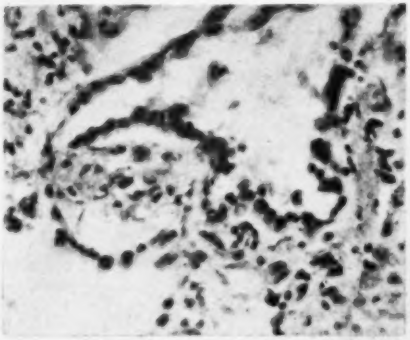

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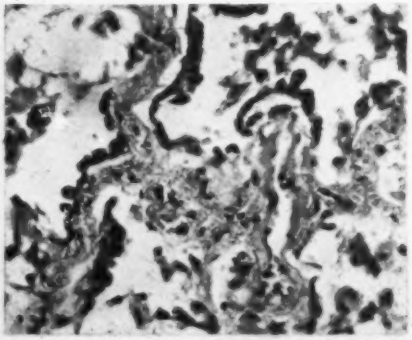

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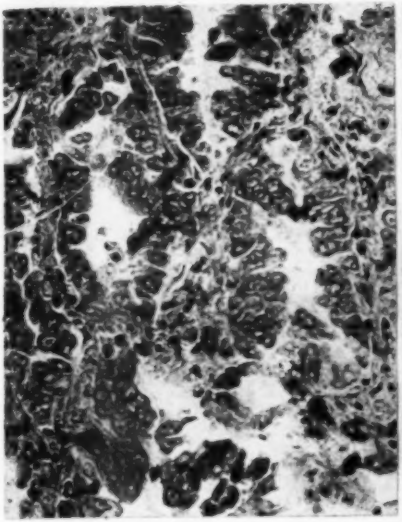

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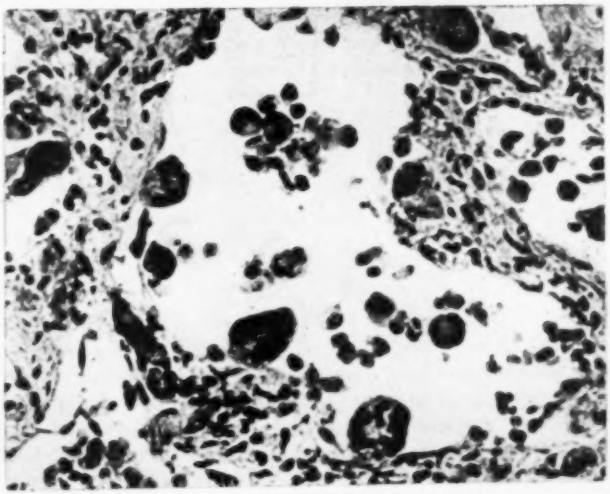

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incidence, 8·85 per cent, of lung cancer in this occupation. The lesions produced in haematite miners are similar to those in coal workers' pneumoconiosis, with comparable simple and massive lesions and focal emphysema (Stewart and Faulds, 1934). The lungs are usually reddish or chocolate coloured.

Metal workers (*Table III*).—Sections from fibrosed areas in lungs from sixteen cases, fourteen with carcinoma of a main bronchus, were examined. Metal grinders are said to have a two and a quarter-fold increased incidence of lung cancer (Kennaway and Kennaway, 1936). McLaughlin *et al.* (1950) found three cancers in sixty-four autopsies of iron and steel foundry workers. Other occupations with similar mixed exposure to iron and silica show a very doubtful increase.

TABLE III.—*Metal Workers* (16 ; 14 *with Carcinoma*)

Occupation	Total	Carcinoma
Steel grinders	5	3 epidermoid 1 adenocarcinoma 1 squamous
Brass workers	3	3 epidermoid
Metal milling machinists	2	1 epidermoid 1 anaplastic
Metal buffer	1	Epidermoid
Metal chipper	1	,,
Tool maker	1	,,
Crane operator over molten metals	1	,,
Iron fettler	1	Nil
Tin worker	1	,,

Painters (Table IV).—The table shows details of occupation and type of carcinoma. Lungs from one case were examined and histological sections from two others, all with carcinoma of main bronchus. Wynder and Graham (1951) suggest that there is some slight evidence for suspecting an increased incidence of lung cancer in this occupation.

TABLE IV.—*Painters* (3—*All with Carcinoma*)

Occupation	Total	Carcinoma
House painters	2	2 epidermoid
Railroad car painter	1	Squamous

Group IV : Industries with increased cancer incidence

Asbestos (Table V).—Table V shows the mode of exposure and type of carcinoma present. Lungs from sixty unselected cases were examined, thirteen with associated carcinoma. Multiple sections were available in each case from areas of local and general fibrosis. There is general agreement in this country that asbestosis is associated with an increased cancer incidence. Gloyne (1951) found 14 per cent ; the *Annual Report of the Chief Inspector of Factories* (1955) gives it as 16 per cent ; and Doll (1955) as 14·2 per cent.

Chromates.—Lung sections from nine chromate workers were examined. Seven cases had developed carcinoma, six anaplastic and one epidermoid. These had all worked in an American plant extracting chromium compounds from iron chromite ore. Their length of exposure varied from four to twenty-five years. The incidence of respiratory carcinoma in this industry is twenty-nine times the

TABLE V.—*Asbestos* (60 ; 13 *with Tumours*)

Occupation	Total	Carcinoma
Asbestos unspecified	22	1 anaplastic 2 epidermoid
Weavers	7	1 epidermoid
Miners	6	1 squamous
Fabricating workers	5	1 adenocarcinoma 1 endothelioma of pleura
Pipe makers	4	1 epidermoid
Mill workers	4	1 adenocarcinoma 1 alveolar cell tumour
Bagger	3	1 anaplastic
Crushing room	3	1 squamous 1 endothelioma of pleura
Carding room	4	—
Waste sorters	2	—

crude mortality rate for all males in the United States (U.S. Public Health Service Publication, 1953). Machle and Gregorius (1948) state that 21 per cent of all deaths in the United States chromate-producing factories are due to carcinoma of the lung. There is doubt as to the occurrence of specific fibrosis in this occupation though bronchitis is common (U.S. Public Health Service Publication, 1953). With the exception of the two non-tumour cases material was limited to existing sections consisting mainly of tumour and not specifically areas of fibrosis.

Nickel.—Nickel workers are thought to show an increased incidence of lung cancer, the *Annual Report of the Chief Inspector of Factories* (1949) mentions eighty-two cases occurring between the years 1923 and 1948 inclusive. Lungs from three cases were available for study, one of which had developed a squamous carcinoma of a major bronchus. Multiple sections were examined from areas of fibrosis. The three men had worked in the same plant where nickel is extracted from the ore by the nickel carbonyl process. Cases 1 and 2 had no definite evidence of exposure to the gas nickel carbonyl ; Case 3, however, had one mild episode of nickel carbonyl poisoning one year before death. All workers had been exposed to mixed dust during refining of the ore.

RESULTS

Age

The overall inception rate of alveolar metaplasia typical or atypical, with or without carcinoma, may be seen in Fig. 2.

One case of berylliosis aged 7 is not included and cases in the eighth and ninth decade are grouped together because of their small number.

It is apparent that the incidence of alveolar metaplasia in all groups combined decreases with age.

Incidence

The total population examined can be subdivided into those with and those without carcinoma, and according to occupation with and without carcinoma. The presence of alveolar metaplasia in each group was determined.

The crude rates of alveolar metaplasia in these sub-populations are not comparable owing to the differences in the age distributions of the various populations. When, by standardisation, we allow for such age variation, and so obtain

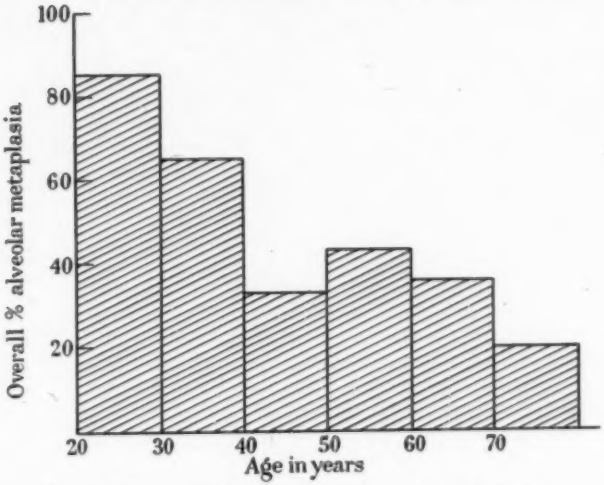

FIG. 2.—The overall inception rate of alveolar metaplasia.

the standardised inception rate, then a true comparison of the overall rates for each subgroup may be made (Table VI). Group III is the combination of all cases in each of its four subgroups. In Group IV the asbestos cases only are included as the ages of the chromate workers were not available and the number of nickel cases was very small (three).

TABLE VI.—*Standardised Inception Ratios (S.I.R.) for Alveolar Metaplasia*

(All subgroups combined = 100)

	Without carcinoma					With carcinoma				Rate for all groups per 100
	Coal	Beryllium	Group III	Asbestos	Total	Coal	Group III	Asbestos	Total	
Number of persons	100	45	22	38	205	30	10	7	47	41·4=100
S.I.R = standardised rate expressed as % of rate for all groups	38	112	200	150	90	91	188	167	136	—
Standard error of above	10·11	21·16	70·75	30·63	10·49	28·73	48·38	74·67	24·90	—

Table VI shows that the rate for all populations surveyed was 41·4 per 100 persons and for the total population without carcinoma it is 90 per cent of this as compared with 136 per cent for all persons with carcinoma. This difference, while not statistically significant, accounted for by the small number of cases with carcinoma, is somewhat suggestive of an increased incidence of alveolar metaplasia in cases with developed carcinoma.

The only group that shows a significant difference in the rate of alveolar metaplasia as compared to the rate for all cases in the survey is coal workers

without carcinoma ; this rate is significantly low. That is, alveolar metaplasia in coal workers without carcinoma is less frequent than was found in all groups combined.

If one compares the rate of alveolar metaplasia in each group in the class without carcinoma with the rate for the whole class it is found that the difference in the rate for asbestosis approaches a significant level (difference 60 ± 32). The incidence of alveolar metaplasia in asbestosis without carcinoma tends to be greater than the incidence for the whole class without carcinoma. The difference in the other groups without carcinoma does not even approach a significant level.

Similarly, we can compare the rate for any one group in the class with carcinoma to the rate for the whole class, the difference is in no instance significant.

The rate of alveolar metaplasia in any one group without carcinoma compared to the rate for that group with carcinoma is in no instance significant. In coal workers the difference approaches significance. This suggests that the incidence of alveolar metaplasia in coal miners with carcinoma is higher than the incidence in coal miners without carcinoma.

Whilst determining the presence of alveolar metaplasia the extent of this change was also observed. It was found that irrespective of occupation or the presence of carcinoma it was most extensive in those cases with diffuse fibrosis. Two of the groups, berylliosis and asbestosis, show diffuse fibrosis as a constant feature and in these alveolar metaplasia when present was very extensive.

Morphology

In Group I (coal workers) the morphology of alveolar metaplasia was identical in the presence or absence of carcinoma. The metaplasia was not particularly related to areas of fibrosis, the involved air spaces being mainly perivascular, peribronchial, septal or subpleural. In no case did the metaplasia show evidence of malignancy, transition to malignancy or continuity with established tumour. The cells were mainly cuboidal, with minimal irregularity of size and shape, and a great dearth of mitosis. They were arranged in a single layer, with no papillary areas. The features were those constituting "typical" alveolar metaplasia (Fig. 3).

In Group II (berylliosis) the features were similar to Group I, typical alveolar metaplasia (Fig. 4).

Group III (oil, haematite, metal and paint workers) showed a similar appearance and distribution apparently unconnected and unrelated to carcinoma. One haematite miner with endothelioma of the pleura showed no typical alveolar metaplasia but many subpleural alveoli were lined by malignant cells in continuity with the tumour. This appearance (Fig. 5) closely resembles alveolar cell carcinoma but the tumour was thought to originate in the pleura.

In Group IV (the asbestos workers with alveolar metaplasia) with two exceptions, showed the typical variety. This was mainly at the margins of secondary lobules and unrelated to developed carcinoma. It is emphasised that atypical alveolar metaplasia was not present in any of the cases with developed carcinoma. The two exceptions which showed atypical metaplasia will be considered individually, as will the case of alveolar cell carcinoma.

Case 27.—Male, aged 53, asbestos miner 19 years. No carcinoma. Moderate degree of interstitial fibrosis with asbestos bodies. There was no evidence of infection or chronic venous congestion in the sections examined. The alveolar

metaplasia was not extensive and was related to septa and areas of fibrosis. It was considered slightly atypical in that some areas showed more than one layer. The cells tended to be large with considerable hyperchromatism but no evidence of mitosis (Fig. 6). These spaces may be peripheral bronchioles and not alveoli but the epithelium is atypical for either.

Case 32.—Male, aged 62, asbestos pipe worker with mixed exposure to silica and asbestos. No carcinoma. Moderate degree of diffuse fibrosis with asbestos bodies, areas of caseous tuberculosis and classical whorled silicotic nodules. In one area, unrelated to tuberculosis or silicosis there was somewhat atypical alveolar metaplasia. Instead of the usual regular cuboidal epithelium, the cells varied in size, shape and staining and some can be seen desquamating into the alveolar spaces (Fig. 7). This slightly atypical appearance is probably associated with the oedema and represents formation of macrophages.

Case 58.—Female, age unknown, asbestos mill worker. Alveolar cell carcinoma. The lungs showed generalised fibrosis with scanty asbestos bodies. The alveolar spaces were lined by non-mucin-secreting columnar cells showing scanty mitosis (Fig. 8). In some areas these cells were multilayered. In the absence of an extrapulmonary primary neoplasm or carcinoma of main bronchus this condition is considered as alveolar cell carcinoma of the diffuse type, involving the whole lung. There was no metaplasia or evidence of a primary carcinoma in the major bronchi. The usual cuboidal alveolar metaplasia was absent.

Group IV, chromate workers, showed four instances of typical alveolar metaplasia, three in lungs with carcinoma of major bronchi and one without carcinoma. The epithelium in all cases was identical and consisted of a single layer of uniform cuboidal cells. There was no continuation or connection with the established bronchial tumours. Due to the paucity of material the relationship to fibrosis could not be evaluated.

Group IV, nickel workers, consisted of three cases which will be considered individually.

Case 1.—Male, aged 58, nickel worker 30 years. No exposure to nickel carbonyl. Chronic bronchitis 30 years. Epidermoid carcinoma. This man had an epidermoid carcinoma of the right upper lobe bronchus, a slight degree of interstitial fibrosis and " compensatory " emphysema. There was no evidence of alveolar (or bronchial) metaplasia.

Case 2.—Male, aged 34, nickel worker 4 years. No exposure to nickel carbonyl. No tumour. The lungs showed widespread fibrosis, bronchiectasis, bronchiolectasis and " cystic change ". The " cysts " were lined by epithelium designated as atypical alveolar metaplasia. This consisted of simple cuboidal cells and scattered bizarre giant-cells, some of which lay free in the air spaces (Fig. 9). The metaplasia was not considered malignant and was of the type sometimes associated with long-standing infection and fibrosis.

Case 3.—Male, aged 58, nickel worker 17 years. One mild attack nickel carbonyl poisoning one year before death. Alveolar cell carcinoma. The lungs showed widespread interstitial fibrosis, bronchiolectasis and " cystic " change. The " cysts " were lined by very atypical squamoid cells amounting to carcinoma (Fig. 10). As this epithelium was lining peripheral air passages in the absence of a primary tumour of main bronchus or other organ it was considered as an example of alveolar cell carcinoma. Typical alveolar cell metaplasia was to all intents absent and there was no transition from one to the other.

Infection

The relation of infection to the presence of alveolar metaplasia in the different groups (*a*) with carcinoma, (*b*) without carcinoma, may be seen in Table VII. Infection includes both pyogenic and tuberculous inflammation. The age incidence is not considered in this and the following Table VIII, so Group IV thus includes asbestos, chromates and nickel workers. The difference in the percentage of cases with and without infection was analysed. This reached a significant level in one instance, Group IV without carcinoma, i.e. those cases in Group IV with alveolar metaplasia, in the absence of carcinoma, show an increased incidence of infection. With this one exception infection does not apparently predispose to the development of alveolar metaplasia.

TABLE VII.—*The Relation of Infection to Presence of Alveolar Metaplasia*

Occupational group	Alveolar + Metaplasia 0	(*a*) With carcinoma		(*b*) Without carcinoma	
		Number of cases	% infection	Number of cases	% infection
I	+	10	90	13	61
	0	20	60	87	67
II	+	—	—	35	28
	0	—	—	17	47
III	+	16	81	8	100
	0	7	71	2	50
IV	+	10	50	34	82
	0	12	50	16	37

Chronic venous congestion

Table VIII shows the relationship of chronic venous congestion, including oedema, to the presence of alveolar metaplasia, (*a*) with carcinoma, (*b*) without carcinoma. The difference between the incidence of chronic venous congestion in those cases with and without alveolar metaplasia is in no instance significant. Chronic venous congestion does not therefore appear to predispose to alveolar metaplasia.

TABLE VIII.—*The Relation of Chronic Venous Congestion (Including Oedema) to Presence of Alveolar Metaplasia*

Occupational group	Alveolar + Metaplasia 0	(*a*) With carcinoma		(*b*) Without carcinoma	
		Number of cases	% C.V.C.	Number of cases	% C.V.C.
I	+	10	60	13	61
	0	20	30	87	48
II	+	—	—	35	40
	0	—	—	17	41
III	+	16	12	8	12
	0	7	0	2	50
IV	+	10	30	34	35
	0	12	33	16	18

DISCUSSION

The results show that the frequency of alveolar metaplasia decreases with age (Fig. 2). It thus does not parallel the increasing incidence of lung cancer with advancing age (Korteweg, 1951). This evidence is therefore against alveolar metaplasia being related to the development of lung cancer.

A comparison of the incidence of alveolar metaplasia in various occupations provides information as to whether the metaplasia is related to the development of carcinoma.

If alveolar metaplasia is premalignant then the frequency might be expected to be greater in the class " with carcinoma " than in the class " without carcinoma ". A statistical test shows that there is no significant difference in the standardised inception ratio of these two classes Similarly, if one considers a single occupational group the incidence might be expected to be greater in those cases with developed carcinoma than in those without carcinoma. The results show that the difference is not statistically significant. Thus, the association of fibrosis, alveolar metaplasia and carcinoma is no more frequent than the association of fibrosis and alveolar metaplasia. Another postulate was also tested : if alveolar metaplasia is premalignant then it might be expected to be commoner in occupations with a high incidence of cancer, e.g. asbestos workers, than in low cancer incident occupations, e.g. coal workers. The only results in favour of this postulate were that in the class " without carcinoma " the rate of alveolar metaplasia in asbestos workers tended to be greater than the rate for the whole class and that of coal workers was lower than the rate for all cases in the survey.

It is thought that the balance of evidence from incidence study does not favour a relationship between alveolar metaplasia and the development-of carcinoma.

The presence of alveolar metaplasia, with one exception, could not be correlated with infection or chronic venous congestion. The high incident tumour occupations, Group IV, without carcinoma showed a significantly high association of alveolar metaplasia with infection. It was important to assess these features as the incidence of alveolar metaplasia was expected to be increased in cases with carcinoma, and might have been accounted for by the frequent complication of infection. The evidence from this study suggest that with the one exception, infection and chronic venous congestion do not predispose to alveolar metaplasia. This is at variance with the work of Gazeyerli (1936) and Geever, Neuberger and Davies (1943).

If alveolar metaplasia is related to the development of carcinoma then one might expect to find them in histological continuity. In no section examined was there any evidence of continuity.

The morphology of alveolar metaplasia, with three exceptions was typical, with no suggestion of malignancy. Two of the atypical type occurred in asbestos workers and one in a nickel worker, but were not thought to be premalignant. One asbestos and one nickel worker showed alveolar cell carcinoma. In 278 cases examined there were thus only two instances of definite malignant disease arising in respiratory air passages. In no instance was there any evidence that the morphology of alveolar metaplasia was related to the presence of carcinoma of the bronchus, in fact atypical alveolar metaplasia did not occur in the presence of main bronchus carcinoma. There was also no transitional type of epithelium between alveolar metaplasia and bronchial or alveolar cell carcinoma. It is

concluded that alveolar metaplasia on morphological grounds is not related to the development of carcinoma. This is in keeping with Gloyne's (1951) conclusion that he could not demonstrate any premalignant changes in asbestosis, and refutes Hutchinson (1952) who suggested that alveolar metaplasia is a precursor of alveolar cell carcinoma.

Barnard and Day (1937) thought that the finding of cuboidal metaplasia in alveoli adjacent to scars was accounted for by their relative fixity. This is partially supported by its frequent presence at the margins of secondary lobules, but the fact that alveolar metaplasia was seen adjacent to " scars " in only 14 per cent of coal workers strongly rebuts this theory. These scars presumably, are as fixed as any other scar. The evidence, thus tends to refute the " fixation " aetiology of alveolar metaplasia.

The hypothesis of Raeburn and Spencer (1953) that many hilar carcinomas may arise in the peripheral air passages appears questionable. In support of their theory they quote the findings of unsuspected microscopic tumours arising in peripheral scars. The results of this investigation do not support their theory. It may be stressed that no localised tumour was found in peripheral scars in any of the industrial lungs examined. It is indeed surprising that inhaled carcinogens do not seem to stimulate the 100 square metres of peripheral epithelium but rather choose the few square centimetres of major bronchi. The hypothesis of Macklin (1956) of carcinogens concentrating in the major bronchi by the action of a " mucus raft " is most attractive. Neuberger and Geever (1942) in an extensive review of the literature up to 1940 state that the majority of authors do not incriminate diffuse fibrosis as a cause of alveolar cell neoplasia. The present findings show that diffuse fibrosis favours the production of benign cuboidal alveolar metaplasia but not alveolar cell neoplasia. This does not imply that diffuse fibrosis is not a " co-carcinogenic " factor in the development of lung cancer. It may be the factor explaining the greater cancer incidence in asbestosis as compared to coal workers.

SUMMARY

The epithelium lining respiratory portions of the lung adjacent to areas of fibrosis was examined in 278 cases, and its relationship to the development of carcinoma ascertained.

Cases were selected from occupations with different incidence of lung cancer varying from low to high and included coal, beryllium, oil, tar, haematite, metal, paint, asbestos, chromate and nickel workers. In each occupation, when available, cases with and without carcinoma were examined.

Alveolar metaplasia was the term applied to cuboidal epithelium lining respiratory air spaces, and was subdivided into typical and atypical.

The overall inception rate of alveolar metaplasia decreased with age.

Alveolar metaplasia in the lungs from occupations with a high incidence of cancer did not show any conclusive difference in frequency or morphology from that seen in occupations with a low incidence of cancer.

The presence of an established carcinoma was not related to the incidence or morphology of alveolar metaplasia.

Atypical alveolar metaplasia occurred in only three cases, two asbestos workers and one nickel worker. The three cases, though atypical, were not malignant or thought to be premalignant. In the 278 cases examined there were only two

carcinomas, alveolar cell, thought to arise in the respiratory air passages, one in an asbestos worker and one in a nickel worker.

Alveolar metaplasia was not particularly related to isolated scars. Irrespective of occupation diffuse fibrosis of the lung predisposed to alveolar metaplasia. "Fixity" of lung scars was not thought to predispose to alveolar metaplasia. No localised tumour was found in any of the hundreds of peripheral scars examined.

The frequency of alveolar metaplasia is not related to the presence of infection or chronic venous congestion.

CONCLUSION

Cuboidal epithelium, alveolar metaplasia, as seen in alveoli and peripheral respiratory air passages, is thought to be unrelated to the development of pulmonary carcinoma.

I am indebted to the British Empire Cancer Campaign for an American-British Exchange Fellowship, 1954–55.

I wish to express my warm thanks to many American pathologists and clinicians for their kind interest and for access to their material and records, in particular Drs. A. A. Liebow, B. Castleman, G. W. H. Schepers, A. J. Vorwald, H. L. Hardy and G. Lindskog.

My thanks are due to Professor J. Gough for his valuable advice and criticism. Also to Dr. Lewis Fanning for the statistical analyses and to Dr. W. R. L. James for access to his material.

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THE SIGNIFICANCE OF CELL TYPE IN RELATION TO THE AETIOLOGY OF LUNG CANCER

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Received for publication December 24, 1957

KREYBERG (1952, 1954*a*, *b*, *c*, *d*, and 1955) has concluded that the histological types of lung cancer to be seen in Norway can be divided into two aetiologically distinct groups. The squamous cell, large cell and small cell carcinomas (including oat-cell carcinomas) form the first group. These types he thought might be histological variants of a single oncological entity and be produced largely by exposure to an external factor (or factors) which have recently increased in prevalence and to which men are principally exposed. The second group is heterogeneous and consists of adenocarcinomas, bronchiolar (alveolar cell) carcinomas and various grades of adenoma and salivary gland type tumours (including cylindromas). These different types, it was suggested, might be produced by different aetiological factors including, perhaps, developmental abnormalities, virus infection and external factors of approximately constant prevalence to which men and women are exposed equally.

In Norway, Kreyberg (1955) estimated that four out of every five Group I tumours in men were related to tobacco smoking, but Group II tumours were found to be entirely unrelated to tobacco. In the U.S.A., Wynder and Graham (1950) had previously obtained evidence suggesting that smoking was more closely related to the development of "epidermoid" tumours than of adenocarcinomas and Wynder (1954) in data collected from several countries found that adenocarcinomas constituted a higher proportion of lung cancer cases among male non-smokers than among male smokers. In Britain, Doll and Hill (1952) found no statistically significant differences in either sex between the amounts smoked by patients with (i) squamous carcinoma (475 men and 18 women), (ii) oat-cell or anaplastic carcinomas (303 men and 38 women), (iii) adenocarcinoma (33 men and 10 women) and (iv) carcinoma of unclassified type (105 men and 13 women). Neither was there any significant difference in the amounts smoked between the four groups of patients in whom histological examination was made and the patients not examined histologically (441 men and 29 women). They noted, however, that in both sexes there were relatively more non-smokers and very light smokers in the small group of patients with adenocarcinoma than in any of the other groups, but the numbers were too few to be convincing.

In the present study these British data have been re-examined in greater detail. Additional evidence has been obtained about some of the cases which could not previously be allocated to one or other histological group and many available specimens have been re-examined. Because of the large number of patients in the whole series a selection for re-examination was made as follows :— (i) all tumours in women, (ii) all tumours in men provisionally classified to Group

II, (iii) all tumours provisionally classified to Group I among men who were non-smokers or who had smoked an average of less than 5 g. of tobacco a day for the previous 10 years, and (iv) a random sample of the remainder. Specimens for re-examination were not available for all these selected cases but with the generous co-operation of many pathologists it was possible to collect a large proportion of the slides and to examine them in the Institute for General and Experimental Pathology in Oslo. In total, sections of 166 tumours were collected—59 out of the 77 female cases which had been classified histologically, 24 out of the 51 male cases provisionally classified to Group II and 83 out of the 107 selected male cases provisionally classified to Group I. Over half the cases for which sections were not available (36/69) had been examined in one hospital department which had been destroyed in a fire; unfortunately they included a disproportionately large number (20) of the Group II cases in men.

To avoid any bias which might result from a knowledge of the sex of the patient or of the smoking history, all identification marks on the slides were covered and each slide was given a code number. The histological classification could, therefore, be made by one of us (L. K.) without any knowledge of the case, save that it had previously been diagnosed as lung cancer.

In Table I the daily amounts smoked by patients with Group I and Group II type tumours are compared with the daily amounts smoked by their control

TABLE I.—*The Amount Smoked by Men and Women with Different Histological Types of Lung Cancer Compared with the Amount Smoked by Control Patients with Other Diseases, of the Same Sex and Age Distribution*

Type of lung cancer	Number of non-smokers	Number smoking an average daily amount during 10 years preceding the onset of the illness of: Less than 5 g.	5–14 g.	15–24 g.	25 g. or more	Total number of patients
Men:						
Group I—						
Number observed	3	29	291	301	208	832
Number expected from experience of control patients	37·8	77·3	345·9	267·4	103·6	832·0
Group II—						
Number observed	2	2	14	16	6	40
Number expected from experience of control patients	1·9	3·6	16·2	13·1	5·2	40·0
Type uncertain—						
Number observed	0	1	2	3	0	6
Women:						
Group I—						
Number observed	16	8	9	15		48
Number expected from experience of control patients	24·9	12·1	8·2	2·8		48·0
Group II—						
Number observed	5	2	4	2		13
Number expected from experience of control patients	7·1	2·7	2·5	0·7		13·0
Type uncertain—						
Number observed	7	1	5	2		15

patients of the same sex and age distribution. It will be noted that the total number of cases differs from that given by Doll and Hill (1952) in their Table X, since the additional information obtained has enabled some of the cases previously described as " carcinoma of unclassified type " to be allocated to one or other of the histological groups; the remaining unclassified cases have been excluded from the study. For the female cases and the male Group II cases the histological grouping is that determined after the review of the available material (by L. K.). For the remainder it is that derived from the routine reports of the pathologist at the hospitals at which the patients were treated since, as stated above, these cases were not all subjected to review. This lack of review is unlikely, it will be shown later, to have seriously affected the results, though it may have somewhat *diminished* the observed differences between the patients in the two groups. In the present series, it may be noted, the cases classified in Group II are more homogeneous than those in Kreyberg's series from Norway (Kreyberg, 1955), since cases described as adenomas, cylindromas etc. had been excluded from the original " lung cancer " series. The group, therefore, largely consists of adenocarcinomas and bronchiolar (alveolar cell) carcinomas; it does, however, include some cases which had originally been described as adenocarcinomas which can also be described as malignant adenomas or salivary gland type tumours.

The control patients used for the comparison were the " matched control " patients (1357 men and 108 women) of Doll and Hill's study (Doll and Hill, 1952). Each of these patients had been selected to be of the same sex and within the same 5 year age group as a lung cancer patient and was treated in the same hospital (or in a similar regional hospital) at or about the same time. The age distributions of the groups of patients with the various histological types of growth are not, however, identical with the age distribution of all the control patients and since the amount smoked varies with age it is necessary to standardize for age. The method of standardization used was as follows. There were 832 male patients with Group I lung cancer, and of these 321 were aged 45-54 years. Among the 1357 male control patients there were 493 in the same age group: 20 were classified as non-smokers, 27 as having smoked an average of less than 5 g. a day, 197 an average of 5–14 g. a day, 176 an average of 15–24 g. a day and 73 an average of 25 g. or more a day. It was therefore calculated that among 321 control patients (the same number as in the corresponding lung cancer group) there should be $\frac{321}{493} \times 20$ non-smokers, i.e. 13·02. The corresponding numbers of control patients in the other smoking categories and in the other age groups were calculated in the same way and the numbers in each smoking category added for all the age groups. The totals thus obtained gave the numbers of patients in each smoking category in a control population of the same size and age distribution as the male patients with Group I lung cancer and could be compared directly with them. The expected numbers for comparison with the other groups of lung cancer patients were obtained similarly.

From Table I it is clear that the distribution of smoking habits in the men with Group I tumours is entirely different from the habits of the control patients. On the other hand there is no appreciable differences between the habits of the men with Group II tumours and the control patients. Among women there is little difference between the habits of the two groups of patients; both groups contained somewhat fewer non-smokers and very light smokers and more moderate

and heavy smokers than the corresponding controls. A statistical test of the significance of the trend of the risk at different levels of smoking shows, however, that it is significant for women with Group I tumours ($P<0{\cdot}01$), but not significant for women with Group II tumours ($P>0{\cdot}1$).

The contrast between the amounts smoked by men with the two types of tumours is demonstrated more clearly in Table II, which shows for each smoking

TABLE II.—*Estimated Relative Risks of Developing Different Histological Types of Lung Cancer (Relative to the Risks Among Non-smokers)*

Type of lung cancer	Relative risk among persons smoking an average daily amount of: Less than 5 g.	5–14 g.	15–24 g.	25 g. or more
Men:				
Group I	4·7 : 1	10·6 : 1	14·3 : 1	25·4 : 1
Group II	0·5 : 1	0·8 : 1	1·2 : 1	1·1 : 1
Women:				
Group I	1·0 : 1	1·7 : 1	8·3 : 1	
Group II	1·1 : 1	2·3 : 1	4·1 : 1	

category the estimated risks of developing a Group I or a Group II tumour relative to the corresponding risk among non-smokers. In each case, the risks have been estimated by dividing the observed number of cases by the expected number from the experience of the controls and expressing the resulting ratio in terms of the ratio for non-smokers (e.g. for Group I men the ratio of observed to expected in non-smokers is 0·079 : 1 and in smokers of less than 5 g. it is 0·375 : 1; the latter is 4·7 times the former).

The trend of the risk with the amount smoked is shown, for each type of tumour, in Fig. 1. For Group I tumours the risk increases steadily and sharply until among smokers of 25 or more grammes of tobacco a day it is 25 times the risk among non-smokers; for Group II tumours the trend is far less striking and varies only between 0·5 and 1·2 times the risk for non-smokers. With women the figures suggest a trend in both groups but the number of patients in Group II is only 13.

The results obtained by the re-examination of the sections of a sample of the Group I growths in men (shown in Table III) suggest that the trend with amount

TABLE III.—*Classification After Review of Cases of Group I Lung Cancer Occurring Among Men Smoking Different Amounts of Tobacco*

Classification after review	Number of cases among men smoking a daily average of: Non-smokers	Less than 5 g.	5–14 g.	15–24 g.	25 g. or more	Total number of cases
Group I	2	13	16	14	19	64
Group II	0	2	0	1	0	3
Group uncertain	1	4	4	3	4	16
% confirmed Group I	68%		79%		83%	77%

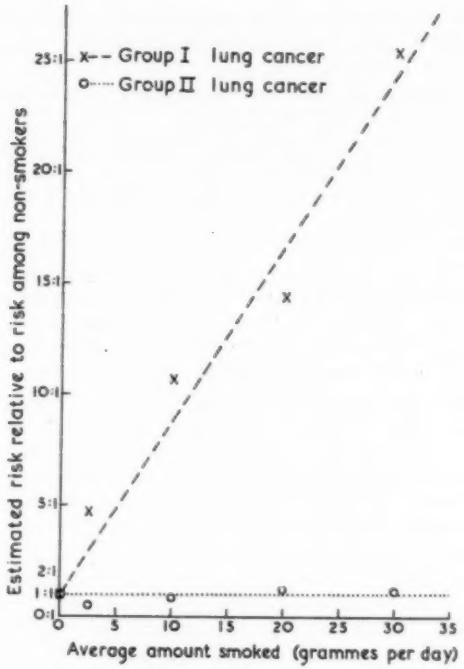

FIG. 1.

smoked may be even sharper than that shown in Tables I and II. No opinion could be expressed on 16 out of the 83 growths examined and the original diagnosis was confirmed in 64 of the remainder. Of the 3 growths which were re-classified as belonging to Group II, two occurred in very light smokers. Thus the proportion of growths confirmed as belonging to Group I, increased from 68 per cent among non-smokers and very light smokers to 79 per cent among medium smokers and to 83 per cent among heavy smokers.

SUMMARY AND CONCLUSIONS

The histological data of the lung cancer cases reported by Doll and Hill (1952) have been re-examined to see whether the relationship then observed between lung cancer and the consumption of tobacco was characteristic of the histological type.

Additional information was obtained about cases for which the histological type had not originally been reported and a proportion of the growths was re-examined by one pathologist, who was kept in ignorance of the sex and previous smoking habits of the patient.

In men, a close relationship was found between the daily amount smoked and the development of squamous, large cell and small cell carcinomas (including

oat-cell and anaplastic carcinomas), but only a slight, if any, relationship with the development of other histological types—principally adenocarcinomas and bronchiolar (alveolar cell) carcinomas.

The number of cases in women was small and it was impossible to decide whether the same distinction held for them.

The results accord closely with those obtained in a Norwegian series and they support the hypothesis that histological types of tumours in the same anatomical site may have a different aetiology, a hypothesis which is also supported by findings in other countries.

We are most grateful to the many pathologists who have co-operated, by making special histological reports and by loaning a selection of their slides for personal examination.

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ARSENIC CONTENT OF CIGARETTES

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Received for publication December 24, 1956

THE object of this communication is (1) to bring up to date our collection of analyses of arsenic in cigarettes, of which two previous instalments have been published (Daff and Kennaway, 1950; Daff, Doll and Kennaway, 1951). The series now comprises 39 brands of cigarettes from 18 countries. (2) To draw attention to some considerable changes which have taken place in recent years. (3) To record some data upon the partition of arsenic between the stump and ash which were not included in the earlier papers.

The two earlier papers reported analyses of cigarettes obtained during 1948–51 from England, Canada, U.S.A., Norway, France, Rhodesia, Switzerland, Italy, Poland, Austria, Greece and Bulgaria, and the results showed as one passes eastwards in Europe a fairly uniform decrease in the arsenic (As_2O_3) content of a cigarette from as much as 100 μg. to 1 μg. or none. There was a gradation from the arsenic-rich American type of tobacco in Western Europe to the arsenic-poor Turkish type in the East, which accords with Hutson's (1937) valuable account of tobacco culture and commerce in Europe. This was almost certainly due to the admixture of decreasing amounts of tobacco from the U.S.A. with increasing amounts of the locally-grown tobacco classed as "Turkish".

To these countries we have now added five (Finland, Denmark, Germany, Spain, Yugoslavia) from which cigarettes were obtained during 1954–56. The very small amounts in those from Central and Eastern Europe (Germany, Yugoslavia) would be expected; two Scandanavian specimens (Denmark, Finland) differ from the earlier one from Norway in giving very low figures. But in view of the very great fall in the arsenic content of tobacco from the United States one cannot now infer that very low figures indicate that the tobacco was grown in Europe. The most expensive of the two brands from Spain show a high content of the old type.

A popular brand of cigarettes ("Klubi") bought in Helsinki are of about the same length (77 mm.) as an ordinary English cigarette, but of smaller diameter (6 mm. instead of 8 mm.) and consists of from 0·4 to 0·53 g. tobacco enwrapped in a paper which encloses also a cardboard tube about 40 mm. long weighing about 0·25 g. Cardboard is an abundant product of the wood pulp industry of Finland. Such cigarettes contain only from one-third to half as much tobacco as do those of the ordinary American or British brands. The arsenic content of the tobacco is low (5·3 μg./g). Another brand (Tyomies) popular in Helsinki is of the usual form, without a tube, but measures only 60 × 8 mm. and weighs about 0·85 g.

Table I includes also (1) Danish Cigarillos, which are of especial interest because they are smoked by women, and (2) a brand of cigars which are among the smallest (weight 2 g., i.e. about twice that of a cigarette) obtainable in England.

Some other analyses of Turkish tobaccos are quoted below.

Enercan (1954) in a paper entitled "Arsenic Contents of Turkish Tobaccos and Cigarette Blends" in 'The Reports of the Turkish State Monopolies Institutes' of Istanbul records analyses of Turkish tobaccos by the Gutzeit method which show very low figures for arsenic (less than 1 μg./g.) and therefore confirm those given in Table I.

	As_2O_3 μg./g.
Fourteen samples from crops of 1949–50	0·1–0·7
Cigarette blends	Same
Pipe blends	0·4–0·5
Cigar blends Two water-pipe blends	0·2–0·4

Recent changes in the arsenic content of tobacco

Our recent analyses (1956) of one of the most popular brands smoked in Britain (Brand P) show a fall in arsenic (As_2O_3) content from between 25 and 100 μg. per cigarette in 1948–9 to between 1 and 2 μg. (Table II). Similar results on a number of brands have been obtained by the research staff of the Imperial Tobacco Company with whom we are in communication (Weber, 1957). The change appears to be due to the gradual abandonment of arsenical insecticidal sprays by the growers in the U.S.A.

Satterlee (1956) states that the "arsenic content of American-tobacco cigarettes" increased from an average of 12·6 μg. in 1932 to one of 42 μg. in 1950–51, but gives no later analyses.

The Partition of Arsenic between the Ash, Stump and Smoke of Cigarettes

These few results seem worth recording because they are derived from cigarette smoking carried out in the ordinary manner and we have not many data obtained in this way (Table II and Fig. 1). Cigarettes of Brand P have a higher range of

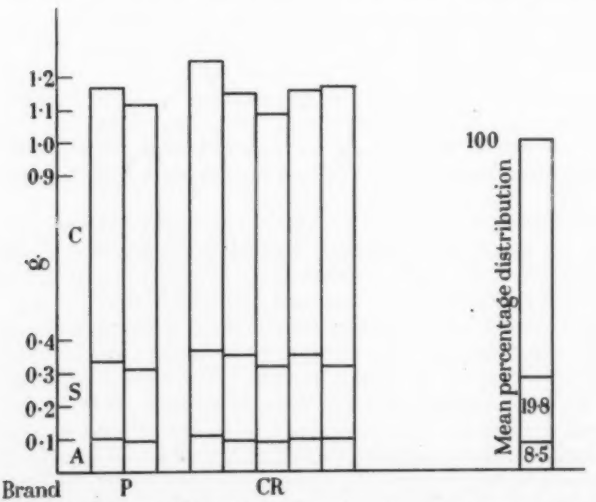

FIG. 1.—Ash and stump of cigarettes. C = cigarette. S = stump. A = ash.

TABLE I.—*Arsenic Content of Cigarettes*

Cigarettes

Country	Year	Brand	Number examined	Weight, g. Range	Weight, g. Mean	As_2O_3 µg./g. Range	As_2O_3 µg./g. Mean
Austria	1950	M	2	0·92–1·06	0·99	0·9–2·1	1·5
Bulgaria	1950	A	4	0·72–0·96	0·84	0·0–1·2	0·6
		R	4	0·85–1·06	0·92	0·25–0·6	0·36
Canada	1950–51	K_1	4	1·03–1·12	1·08	36·8–55·1	48·0
		O	4	1·09–1·16	1·12	8·6–18·7	13·6
		W	6	1·00–1·08	1·03	41·1–79·5	55·7
		Bc	3	1·13–1·18	1·16	42·5–57·6	47·9
		S_1	4	1·05–1·14	1·09	47·4–65·7	57·0
Denmark	1955	K_2	3	1·09–1·15	1·11	61·0–92·0	81·0
England	1948–49	P_1	23	1·08–1·21	1·12	23·9–106·0	54·2
		C_1	23	0·93–1·18	1·00	28·3–61·6	53·0
	1956	P_1	3	1·0–1·1	1·03	1·4–1·8	1·6
	1956	IT	2	1·1–1·2	1·1	1·8–1·9	1·85
England "Turkish"	1948–49	Eight brands	12	0·99–1·04	1·01	0·0–4·3	1·4
Finland	1954	K_2	5	0·41–0·53 Tobacco	0·45	0·0–11·0	5·3
		Cardboard tube	—	—	0·25	—	0·0
France	1949	G	2	1·0–1·18	1·09	0·5–1·5	1·0
Germany	1956	Mc	2	1·03–1·17	1·1	1·5–1·6	1·55
Greece	1951	Pa	2	0·92–1·07	0·99	0·0–1·0	0·5
Italy	1950	N	6	0·85–1·02	0·97	8·3–13·7	10·3
		NM	1	—	0·84	—	0·0
		Sa	1	—	1·15	—	1·5
		M	1	—	0·87	—	Trace
Norway	1950	B	2	1·2–1·3	1·25	65·8–74·6	70·2
		T	2	—	1·0	49·7–55·6	52·6
Poland	1950	Z	5	1·1–1·3	1·2	1·2–2·7	2·0
Rhodesia	1950	R	2	0·96–1·12	1·04	1·8–2·6	2·2
Spain	1956	IM	3	1·2–1·25	1·22	3·2–3·3	3·2
	1956	AC	2	1·04–1·2	1·12	54·0–58·0	56·0
Switzerland	1950	P_2	4	0·89–1·05	0·96	3·2–6·4	4·2
Turkey	1951	—	1	—	1·3	—	0·0
U.S.A.	1950	E	2	0·98–1·06	1·02	41·0–47·0	44·0
		S_2	5	1·03–1·08	1·05	25·2–31·2	27·9
		C_2	2	1·08–1·1	1·09	46·4–46·7	46·5
		P_2	1	—	1·17	—	51·0
Jugoslavia	1956	—	1	—	0·85	—	1·05
Cigars							
Denmark	1955	Cigarillo	4	1·25–1·67	1·55	10·7–22·0	16·0
England	1956	W. W.	1	—	2·01	—	25·8
Jamaica	1956	L.T.	1	—	5·85	—	0·5

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arsenic content than have those of C and this difference appears in the ash. The seven cigarettes were smoked by two persons, the time taken being 10 to 15 minutes. The ash and stump were dropped into weighing-bottles. The figures show :

(*a*) The constancy of the amount of ash (mean 8·5 per cent of the mean weight of the cigarette).

(*b*) The greater variation in the weight of the stump range 0·214 to 0·256 g., the mean being one-fifth of the mean weight of the cigarette. The portion of a cigarette which becomes the stump gains in weight from condensation of smoke. Comparative data from other countries, and especially the U.S.A., would be valuable.

(*c*) The ash of a cigarette contains from 1·5 to 3·6 times as much arsenic as does the stump.

(*d*) The concentration of arsenic in the ash is 5 or 6 times greater than that in the stump.

(*e*) Of the arsenic in a cigarette, from 7 to 18 per cent is volatilised in smoking, while about 60 per cent remains in the ash and about 25 per cent in the stump.

Table II shows also estimations of arsenic (1) on the ash of cigarettes made (1954) by a firm of tobacco manufacturers especially for Dr. A. J. Lindsey and smoked on his apparatus at the Sir John Cass College (Commins, Cooper and Lindsey, 1954) and (2) on cigarettes from the same source made wholly of paper (Cooper and Lindsey, 1954 ; Cooper, Gilbert and Lindsey, 1955). As the paper of a cigarette makes up about 4 per cent of its weight, i.e. about 0·04 g., this amount of such paper would contain about 0·012 μg. of arsenic.

TABLE II.—*The Partition of a Cigarette when Smoked*

Brand (1949)	Smoker. Time (min.).	Weight, g. Cigarette	Stump	Ash	Ash +stump	Total As_2O_3 μg. Stump	Ash	As_2O_3 μg./g. Stump	Ash
P	NMK (15)	1·165	0·233	0·100	0·333	27	40	116	400
	„ (10)	1·113	0·219	0·091	0·310	25	67	114	736
	Mean	1·139	0·226	0·095	—	26	53	115	568
C	NMK	1·244	0·256	0·11	0·366	8	29	31	264
	„	1·144	0·257	0·095	0·352	12	31	47	326
	„ (12)	1·081	0·228	0·091	0·319	10	27	44	296
	IA (12)	1·156	0·252	0·098	0·350	18	36	71	367
	„ (12)	1·166	0·214	0·100	0·314	12	40	56	400
	Mean	1·158	0·241	0·099	—	12	33	50	331
Mean of all		1·15	0·237	0·098	0·335	16	39	68	398
			19·8 per cent of 1·15	8·5 per cent of 1·15	Ratio	1	2·4	1	5·8
Experimental cigarettes (Cooper and Lindsey, 1954)	Tobacco : Batch 1	—	—	—	—	—	—	—	50, 58.
	2	—	—	—	—	—	—	—	125, 125.
	3	—	—	—	—	—	—	—	66.
	4	—	—	—	—	—	—	—	128, 155.
	Paper	—	—	—	—	—	—	—	2·8, 3·0.

SUMMARY

(1) Data are given for the arsenic content of 39 brands of cigarettes obtained from 18 countries during 1948–56. During the earlier part of this period there was a considerable contrast between the high arsenic content of cigarettes composed wholly or largely of American tobacco and the low arsenic content of those consisting of tobacco grown in Eastern countries and classed as Turkish, the range in the whole series being from over 100 μg. As_2O_3/g. to *nil*.

(2) Recently the arsenic content of American cigarette tobacco has fallen very considerably and some cigarettes from Eastern and Western countries may show no difference in this respect.

(3) Some data are given upon the partition of a cigarette when smoked into stump, ash, and smoke.

We wish to express our thanks to the British Empire Cancer Campaign, the Anna Fuller Fund and the Medical Research Council for grants.

We are indebted to various friends, and especially to Mr. H. L. Henderson, O.B.E., who have obtained cigarettes for us during their travels.

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THE ARSENIC CONTENT OF HUMAN LUNGS AND TRACHEO-BRONCHIAL LYMPH GLANDS

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Received for publication December, 24, 1956

Method of estimation of arsenic

THE method chosen is the photometric one of Vašák and Šedivec (1952) which is based on a colour reaction between arsine (AsH_3) and silver diethyldithiocarbamate dissolved in pyridine ; this is very sensitive and can be adapted, by suitable choice of volume of the absorbent, to measure quantities of arsenic ranging from 0·2 μg. upwards.

Technique

The tracheal and bronchial lymph glands were dissected out, homogenised with water and dried on a water bath, and the residue combusted using 5 ml. concentrated sulphuric acid and 25 ml. concentrated nitric acid. The combustion was continued until the solution was almost colourless ; further amounts of nitric acid were added during the combustion. The solution was then diluted with 25 ml. water and boiled until white fumes of SO_3 appeared, to remove nitric acid, and this boiling with water was carried out a second time. Then 25 ml. water was added, 1 ml. potassium iodide (10 per cent) and 1 ml. stannous chloride (40 per cent) and the solution boiled for one minute to reduce any 5-valent arsenic to the 3-valent form. The solution was diluted to 20 ml. with water and the arsenic estimated.

In the first cases examined a portion of each lung was homogenised with water and dried on a water bath ; the residue was ground to a fine powder and 2 g. of powder combusted as described above. In view of the large variations of arsenic content found in different portions of the same lung, it was decided to dissect out the larger bronchial tubes and homogenise the remainder of the lungs. A sample of the homogenate was then dried and about 2 g. of the finely ground powder combusted.

Estimation of arsenic

The silver diethyldithiocarbamate was prepared from sodium diethyldithiocarbamate, $(C_2H_5)N.CS.SNa.3H_2O$, and silver nitrate. Silver nitrate (1·7 g. in 100 ml. water) was added to a solution of sodium diethyldithiocarbamate (2·25 g. in 100 ml. water). The yellow precipitate was filtered off, washed several times with water, and dried over calcium chloride ; 1 g. was dissolved in 200 ml. pyridine, filtered and kept in a brown glass bottle.

The apparatus for evolution of arsine (Fig. 1) consists of a flat-bottomed distillation flask fitted with a glass stopper provided with a funnel which reaches to the bottom of the flask, and an outlet tube. Test tubes are used for the absorp-

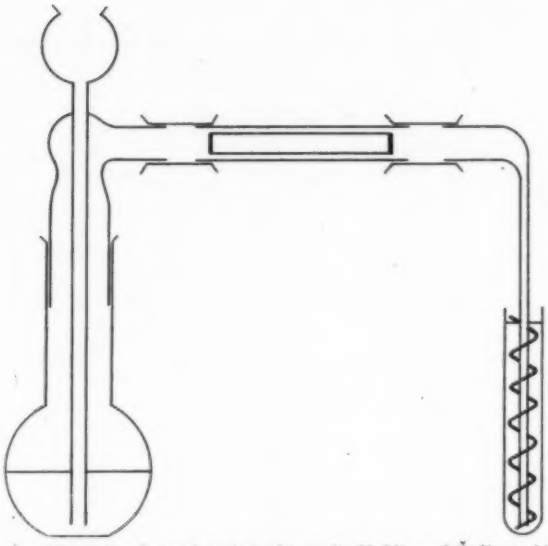

FIG. 1.—Apparatus for estimation of arsenic (Vašák and Šedivec, 1952).

tion of arsine, provided with glass spirals to increase absorption of the arsine by the pyridine solution. The tube connecting the flask and absorption tube contains a paper strip saturated with lead acetate to remove any H_2S produced.

The absorption curves for silver diethyldithiocarbamate in pyridine (A) and for the same after the introduction of arsine (B) were measured on a Unicam spectrophotometer (Fig. 2).

Light absorption is at a maximum at 540 mμ. A standard calibration curve was then constructed. Known amounts of arsenic as Na arsenite were placed in the distillation flask, water added to give a total volume of 20 ml. and 5 ml. concentrated HCl ; 1 ml. 40 per cent stannous chloride and 3 g. zinc were added. The arsine was led over lead acetate paper into 2 ml. reagent, the reaction being allowed to proceed for about 2 hours. The red colour produced was measured, using an EEL colorimeter and a yellow-green gelatine filter 625 ; the instrument was adjusted to zero using the yellow pyridine solution of silver diethyldithiocarbamate. A standard calibration curve was constructed. The solution after combustion of the tissue, and reduction, was placed in the distillation flask, 5 ml. concentrated HCl and 3 g. of zinc added and the estimation carried out as before. The amount of arsenic present was read off from the standard curve.

(1) Table I shows the range of arsenic content of the tracheo-bronchial glands, and lungs, found at autopsies in London on adults, 23 in all, of both sexes of ages ranging from 26 to 87. The cases showed no obvious signs of pulmonary disease, but were otherwise unselected.

(2) The amounts of arsenic in the glands are very small (the highest total was 3·5 μg.) but are high in relation to the small amount of tissue.

(3) The total amount of arsenic in the lungs is about 34 times that in the glands (mean of both sexes).

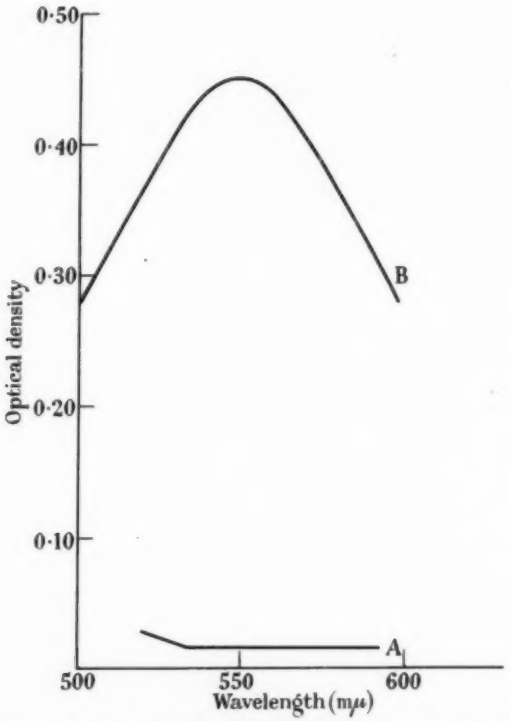

FIG. 2.—Absorption curves of silver diethyldithiocarbamate in pyridine (A) and the same after introduction of arsine (B).

(4) The number of cases is of course much too small for any generalisations but one notes that the only considerable sexual difference is the much higher total in the male lungs (64 : 28). Such a difference would be due in part to the greater mean body weight of men. But other factors (smoking, industrial conditions) might be concerned.

Text-books give a great variety of figures for the average weights of the lungs, which is of course affected by the amount of blood present, in the two sexes. Miller (1947) gives 1058 g. (male) and 924 g. (female) and quotes from C. Krause (1876) the figures 1300 g. (male) and 1023 g. (female). The mean weights in the present series were 1063 g. (males) and 634 g. (female). As in the females the mean amount of arsenic is 28 μg. both in the whole lung and in 100 g. powder, the mean dry weight must be 100 g. which, when derived from a mean wet weight of 634 g. indicates a water content of 84 per cent.

Some Data from Prag

Sula and Zelenkova (1955) record estimations of arsenic in human lungs, liver, spleen, and kidneys, and in the bronchial lymph glands (" anthracotic

TABLE I.—*The Arsenic Content of Lungs and Tracheo-bronchial Glands*

Case No.	Age (Females)	Lymph glands: Total wet weight (g.)	Lymph glands: As_2O_3 µg. Total As_2O_3 µg.	Lymph glands: As_2O_3 µg. As_2O_3/100 g. tissue.	Lymph glands: As_2O_3 µg. As_2O_3/100 g. dry powder	Lungs: As_2O_3 µg. Total As_2O_3	Lungs: As_2O_3 µg. As_2O_3/100 g. dry powder
1	63	5·1	0·9	17·7	—	—	—
2	56	8·2	1·0	12·3	—	—	—
3	73	11·0	1·7	15·5	—	—	—
4	59	5·0	1·7	34·0	—	—	—
10	62	12·2	1·8	14·7	77	58	55
11	46	3·5	0·0	0·0	0	19	17
12	73	13·5	2·2	16·2	67	21	24
13	26	2·6	1·0	38·4	—	24	32
14	54	14·1	2·0	14·0	51	23	20
15	66	13·5	0·8	5·9	34	22	18
	Mean	8·9	1·3	16·9	46	28	28
	(Males)						
16	66	6·0	3·5	58·3	287	88	54
17	71	8·2	1·3	15·8	65	122	45
18	66	7·6	0·6	7·9	38	30	19
19	35	7·1	1·0	14·0	79	110	56
20	69	8·3	1·0	12·0	66	33	29
21	59	22·1	1·9	8·6	49	55	26
22	73	13·3	0·6	4·5	25	24	25
23	77	18·5	0·6	3·2	20	49	15
25	67	19·0	2·4	12·6	—	—	—
	Mean	12·2	1·4	15·2	79	64	34
					Omit No. 16 : 49		
				Mean 14 cases. Males and females	66	48	31
					Omit No. 16 : 48		

						Lungs As_2O_3 µg./100 g. tissue	
	Males					Specimen I	Specimen II
6	87	11·4	1·3	11·4	—	12·6	6·8
7	78	8·9	2·0	22·4	—	2·4	8·9
8	71	5·7	2·6	45·6	—	6·0	20·9
9	67	7·2	1·0	13·9	—	14·6	8·6
All male : Mean		11·0	1·5	17·7			
Mean of 23 cases : male and female		10·1	1·4	17·3			

pulmonary nodes "). As their report, in the Czech language, is not very accessible, a translation of their tabulated results, which have been rearranged, is given in full here (Table II); their figures for As have been recalculated for As_2O_3.

Sula and Zelenkova were able to obtain bronchial glands of varying degrees of "anthracosis" i.e. blackness, ranging from "no" or "slight" to "very strong". We are unable to give any comparable data because bronchial glands from autopsies on adults in London are almost always black; this blackness conceals considerable differences in the amounts of carbon present (Blacklock, Kennaway, Lewis and Urquhart, 1954). In Great Britain adult bronchial glands which are not black are difficult to obtain; Professor D. F. Cappell of Glasgow has kindly undertaken to send us any that come to his notice.

The cases examined by Sula and Zelenkova were presumably adults, as the authors say nothing about age; the anthracosis was "of town and not of mining

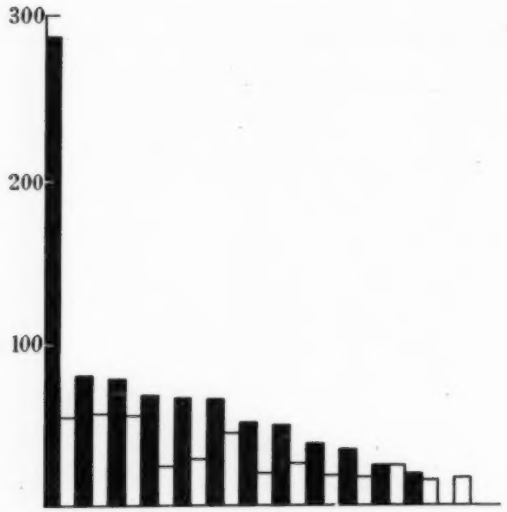

FIG. 3.—Arsenic content of lungs and tracheo-bronchial glands.
(As_2O_3 μg./100g. dried powder.)
□ Lungs. ■ Glands.

origin", but no information is given about sex or social or occupational conditions. One would like to know why the less anthracotic glands from Prag contain on an average more arsenic than do the more anthracotic glands from London, although the amounts of arsenic in the former series are more or less proportional to the degree of blackness.

No totals for the various organs, and no figures based on dry weights are given. The mean concentration in the fresh glands, omitting Case 1, is 39·7 μg., which is more than twice the corresponding figure in Table I, namely 17·3. The authors make no comment upon the extremely high figure (415 μg.) for the glands in Case 1, which is more than 10 times the mean of the remaining 9 cases. The mean concentrations in the lungs, spleen and kidneys are very similar (6·9, 8·3, 6·6);

TABLE II.—*The Arsenic Content of the Lungs, Bronchial Glands and Other Organs (Sula and Zelenkova, 1955)*

	As_2O_3 μg./100 g. wet tissue Case No.										
	1	2	3	4	5	6	7	8	9	10	Mea
Glands	415·3	81·7	69·8	48·3	47·3	29·3	22·7	22·0	21·6	14·3	77·
											Om
											No.
Degree of anthracosis	Very strong	Strong	Strong	Medium	Medium	Slight	Slight	Slight	Slight	None	39·
Lungs	6·6	3·2	6·2	—	6·7	—	9·0	—	11·9	4·4	—
Liver	—	8·5	6·2	—	5·7	—	15·9	6·0	5·7	9·8	6·
Spleen	6·6	6·5	6·0	—	6·2	—	—	5·6	10·5	3·4	8·
Kidneys	—	—	4·4	—	—	—	—	6·8	6·8	8·2	6·

the corresponding figure for the lungs in Table I is 5·4. The liver, which may receive arsenic from the food, shows a higher value (8·3).

We have now obtained in this laboratory data for a comparison of the amounts of carbon, ash (Blacklock *et al.*, 1954) and arsenic in the lungs and bronchial glands (Table III) of adults in London without obvious pulmonary disease. As one might expect, the more soluble arsenic compounds show the least tendency to accumulate in the gland. This indication in regard to arsenic is compatible with the figures from Prag, quoted above, for the content of the lungs, spleen and kidneys.

TABLE III.—*Amounts in Bronchial Glands as Percentage of Total in Lungs + Glands*

	Carbon		Ash		Arsenic	
	Range	Mean	Range	Mean	Range	Mean
Males	4·0–19·2	8·8	6·5–33·3	18·8	1·3–3·8	2·0
Females	5·1–28·0	16·0	10·3–55·6	31·7	0–9·4	4·5

SUMMARY

Data are given for the range of arsenic content of the tracheo-bronchial glands, and lungs, found at autopsies in London on adults, 23 in all, of both sexes, of ages ranging from 26 to 87, showing no obvious signs of pulmonary disease, but otherwise unselected.

We wish to express our thanks to the British Empire Cancer Campaign, the Anna Fuller Fund and the Medical Research Council for grants.

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AROMATIC HYDROCARBONS FROM VEHICULAR EXHAUSTS

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Received for publication December 21, 1956

THE greater frequency of cancer of the lung in urban over rural populations has been well established. This has prompted the evaluation of the aetiological relevancy of agents which have been introduced or which have increased in quantity in urban atmospheres in recent years. Thus, Kotin, Falk and Thomas (1954, 1955), sampling the particulate phase of exhausts from both petrol and diesel engines, identified seven polycyclic hydrocarbons spectroscopically, including the traditional animal carcinogen, 3, 4-benzpyrene, and showed their differential formation with respect to engine speed and load.

The present paper records an assay for aromatic hydrocarbons of a sample of petrol exhaust soot produced under the varying conditions of normal automobile running and compares the results with those obtained from a sample of general atmospheric soot. A sample of diesel exhaust soot, collected as the engine idled, was similarly investigated.

EXPERIMENTAL

The twin exhaust pipes of a 3·6-litre private car were muzzled with fine hemp sacks and an exhaust sample collected over a short period of normal motoring. The sacks and contents were extracted by refluxing with acetone. The acetone extract was taken to dryness and the residue extracted by refluxing for three 3-hour periods with petroleum-ether (B.P. 60–80° C.). The combined extracts were taken to a small volume, and the free carbon of the residual soot estimated. The concentrated extract was fractionated by adsorption chromatography.

The following chromatographic procedure was employed. The concentrated extract, which contained 4·56 g. of extracted material, was layered on top of a 30 × 4 cm. column of alumina (100–200 mesh) and developed with 60–80° petroleum-ether. The progress of the fractionation in this and subsequent chromatograms, was followed by periodic inspection of the columns under a filtered U.V. lamp. All columns were screened from diffuse daylight by wrappings of black paper, to minimize incidental photo-oxidation.

In the initial chromatogram, a fast moving slightly fluorescent oil was detected in the eluate. For the full development of the chromatogram, chloroform was incorporated in the eluent to a concentration of 40 per cent. Thus more strongly adsorbed components were eluted. In all, twelve fractions were collected.

As considerable overlapping of zones occurred, each fraction was separately chromatographed on 20 × 2·5 cm. columns of 100–200 mesh alumina (Spence). The first three fractions were chromatographed using petroleum-ether as eluent. The remaining nine fractions were each developed with petroleum ether containing

2 per cent to 8 per cent acetone. Of various solvent mixtures tried, petroleum-ether containing low concentrations of acetone had been shown to be most suitable for the separation of higher polycyclic hydrocarbons. Discrete zoning is achieved and development is very rapid. The various sub-fractions were purified by rechromatography on 100–200 mesh Silica Gel (Light & Co.) with petroleum-ether as solvent, screened ((*a*) by fluorescence spectrography using a Hilger E.3 quartz spectrograph and excitation radiation 3650 A ; (*b*) by taking absorption spectra in the range 260 mμ–460 mμ. Fractions showing similar spectra were combined and rechromatographed on alumina and silica gel as described above.

Fig. 1 records the fluorescence spectra of compounds separated in the above manner, in approximate order of decreasing solubility. Exposures varied from 3 to 15 minutes. Ilford H.P.3 plates were used initially with ID-11 as developer. The spectra on Fig. 1 were so prepared. However, Ilford " Lond Range Spectrum " plates have been found to give more sharply defined spectra.

According to the relationship noted by Cook, Schoental and Scott (1950), compounds X (Perylene), XII (anthanthrene) and XIII (which has a brilliant green fluorescence) have a minimum of two quinonoid rings, the rest of the compounds having a minimum of one. Compounds XX and XXI (Fig. 1) resemble spectrographically compounds isolated from horizontal retort tar by Berenblum and Schoental (1947), who showed that two fractions which recorded main fluorescence bands at 391 mμ and 385 mμ respectively, were carcinogenic for rabbit skin.

U.V. absorption spectrophotometry confirmed the presence of the compounds identified by the spectrographic method. In addition, the following compounds were identified : pyrene, " Compound X " (Kotin, 1954), 1,2-benzpyrene, 1,12-benzperylene, and coronene. Fig. 2–6 represent the absorption spectra of compounds isolated from the petrol exhaust soot, all of which were more strongly adsorbed on alumina than 3,4-benzpyrene. Identification was rendered difficult as the quantities involved were small and contaminants difficult to separate.

Fig. 2 represents a compound with a brilliant blue-violet fluorescence which occurs in diesel soot, atmospheric soot, and cigarette smoke (Lyons, 1956, Fluorescent Spectrum IV) as well as petrol soot. It gives a characteristic fluorescence spectrum (XIV). It has the following absorption maxima in cyclohexane : 401, 380, 360, 308, 296, 282 mμ.

Fig. 3. This fraction had a violet fluorescence. The fluorescence spectrum is presented (XX). Its absorption spectrum in cyclohexane had the following maxima : 391, 383, 366, 348, 331, 306, 290 mμ. The spectrum resembles that of 3,4-benztetraphene (Clar, 1952, p. 209).

Fig. 4 and 5 both have blue-violet fluorescence and are closely associated chromatographically. Absorption maxima, with benzene as solvent, were : Fig. 4 : 408, 405, 396, 376, 356, 340; 308, 296, 284 mμ. Fig. 5 : 420, 405, 396, 368, 346, 328 (308), 304, 296 mμ.

Fig. 6. This spectrum was given by a fraction which had a blue fluorescence. It is obviously a mixture of two or more compounds. They occurred in too low a concentration to achieve an effective separation. The presence of the potent carcinogen, 3,4,8,9-dibenzpyrene is suggested (Clar, 1952, p. 345). Absorption maxima in benzene were : 452, 424, 402, 380, 314, 300, 290 mμ.

Fig. 7 represents the Log *e* absorption spectrum of a sample of the 3,4-benzpyrene isolated from petrol soot, with a standard sample for comparison.

In the chromatography of both the petrol and diesel soots the aromatic hydrocarbons were preceded by an oil which was weighed following removal of the solvent. This oil was considered to be unburnt fuel and overhead lubricant. It readily dissolved crystalline 3,4-benzpyrene and other aromatic hydrocarbons.

Some of the major occurring hydrocarbons in petrol, diesel and atmospheric soot were estimated by the base-line technique. The results are presented in Table I with reference to parts per million of free carbon. Also included in Table I are the ratios of acetone extracted material, petroleum-ether extract and oil to free carbon.

TABLE I.

	Atmospheric soot	Petrol exhaust soot	Diesel exhaust soot
3,4-benzpyrene (p.p.m. free carbon)	380	1570	20
Pyrene (p.p.m. carbon)	650	440	820
Anthracene (p.p.m. carbon)	215	385	60
1,2-benzanthracene (p.p.m. carbon)	—	180	—
Acetone extract/free carbon	1·06	3·50	0·6
Pet.-ether extract/free carbon	0·17	3·49	0·58
Oil/free carbon	—	0·92	0·29

DISCUSSION

The presence and intensity of absorption maxima, and fluorescence spectra where the requisite reference compounds were available, were the only means employed to identify the aromatic hydrocarbons separated from the various soots. Fluorescence spectrography proved valuable in segregating the various compounds, but sometimes superimposition rendered the interpretation of the spectra difficult. Quantitative differences occurred in the relative concentrations of the hydrocarbons. Chrysene was the only aromatic hydrocarbon occurring in the atmospheric soot which was not detected in the vehicular exhaust soots. On the other hand the brilliant green fluorescent compound (Fig. 1, XII) which is being further purified, occurring in reasonable concentration in the latter, was not detected in the atmospheric soot. The compound (Fig. 1, XIV) found in all three soot samples was relatively diluted in the atmospheric soot. Thus, if vehicular exhausts were shown to be the chief source of emission of this compound into the atmosphere, it is conceivable that its index of dilution in atmospheric soot could be used to gauge the atmospheric pollution component due to vehicular exhausts.

The demonstration of 3,4-benzpyrene as the hydrocarbon of highest concentration of those estimated in the sample of petrol soot is at variance with the data of Kotin, Falk and Thomas (1954), which would place pyrene at higher concentration to 3,4-benzpyrene. The low concentration of 3,4-benzpyrene in the diesel soot, sampled as the engine idled, conforms to the concentrations achieved by Commins, Waller and Lawther (1956).

On comparison (Table I) of the petrol exhaust soot with the general atmospheric soot two significant differences were noted, apart from the possible formation of a highly potent carcinogen like 3,4,8,9-dibenzpyrene and compounds XX and XXI in the petrol internal combustion engine :

(*a*) The relatively high proportion of 3,4-benzpyrene in petrol exhaust soot ; (*b*) the relatively high ratio of petroleum-ether extract to free carbon in petrol exhaust soot, in which was reflected the presence of a considerable quantity of oil (26·4 per cent in petroleum ether extract).

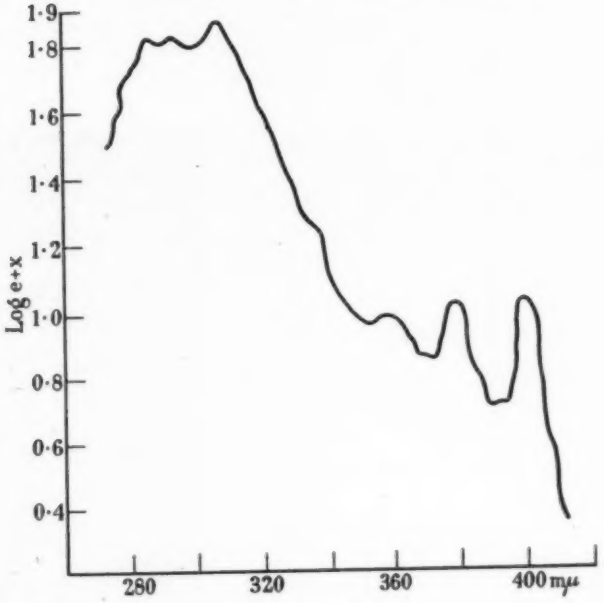


FIG. 2.—See text.

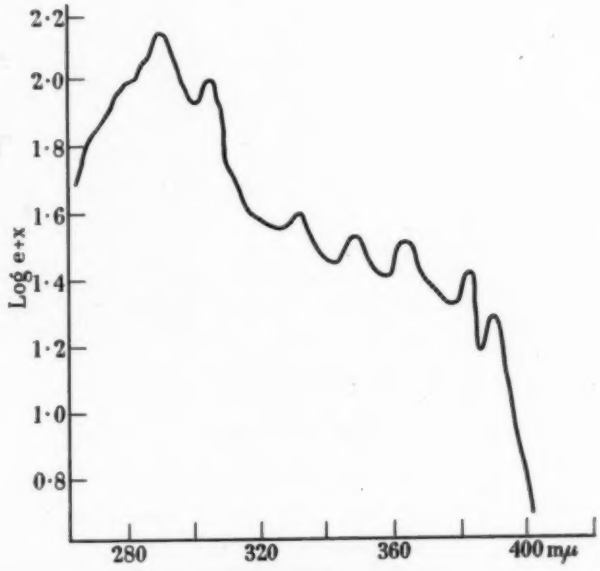


FIG. 3.—See text.

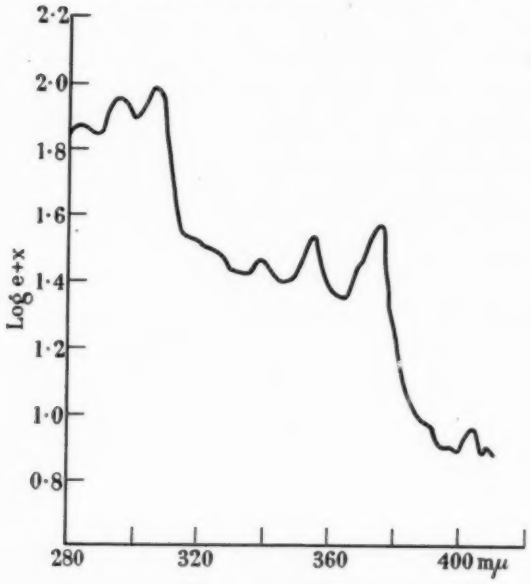


FIG. 4.—See text.

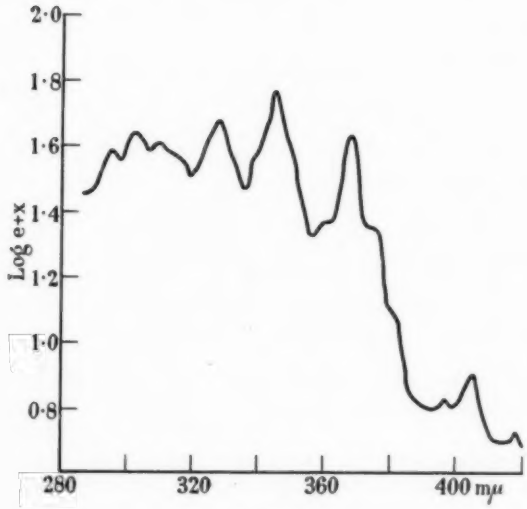
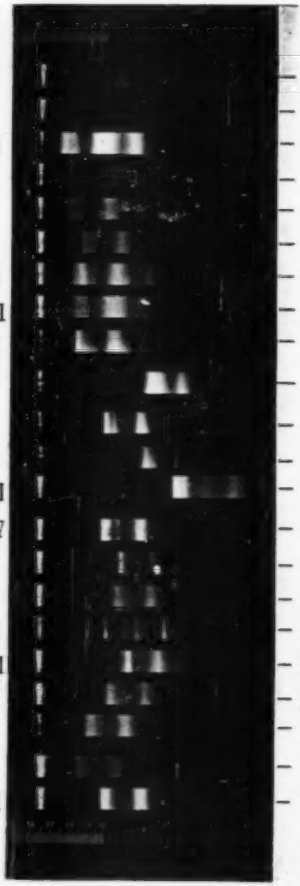


FIG. 5.—See text.

EXPLANATION OF PLATE.

FIG. 1.—Fluorescence spectra of compounds isolated from " petrol exhaust " soot. Solvent, cyclohexane.



	mμ	Compound
I	388,408	
II	398,424,448	
*III	378,395,420	Anthracene
IV	387,407	
V	382,403,428	
VI	390,412,440	
VII	389,(396),410,435	
*VIII	386,403,428	
*IX	385,404,428	1,2-Benzanthracene
X	437,464	Perylene
*XI	403,(408),426,454	3,4-Benzpyrene
XII	428,458	Anthanthrene
*XIII	458,(466)492,526,570	
*XIV	401,(410)427,456	
XV	414,437	
XVI	410,435	
XVII	404,418,428,450,464,470	
XVIII	422,445	
XIX	405,430	
XX	391,414,340	
XXI	385,407,334	
XXII	400,425,455	

* Occurred also in the diesel exhaust soot

Fig 1.

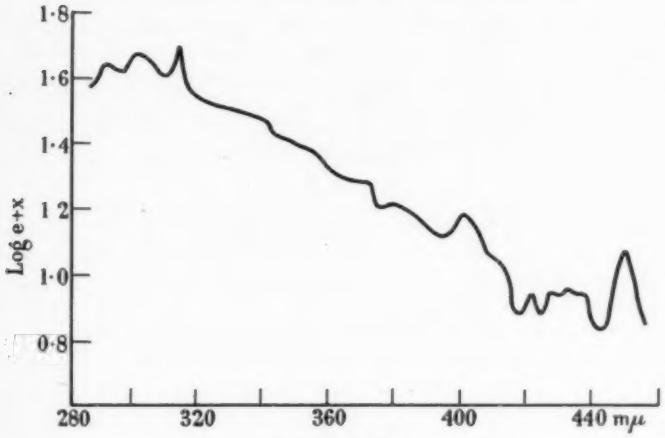


FIG. 6.—See text.

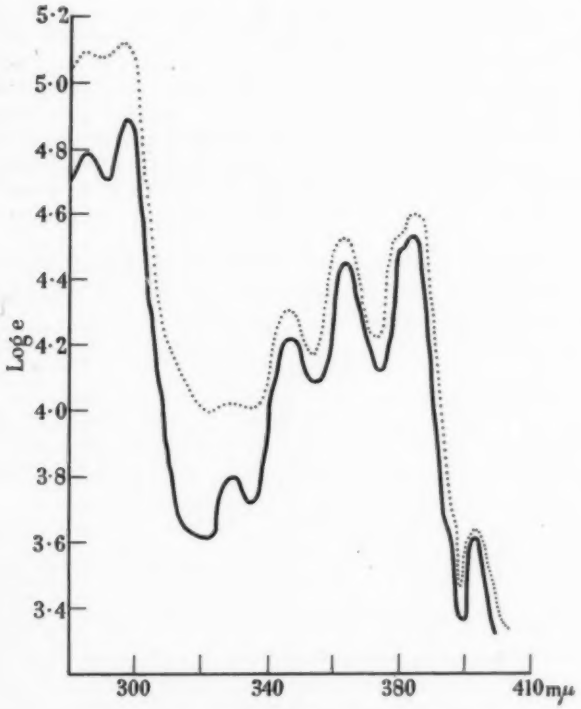


FIG. 7.

——— Absorption spectrum of purified commercial 3,4-benzpyrene.
..... Absorption spectrum of 3,4-Bp. sample isolated from " petrol exhaust " soot.

Ingalls (1950) has pointed out the possible great difference in carcinogenic hazard between soots of high tar content and soots such as industrial carbon blacks with a low tar content. However, experimentally, three main conditions determine the carcinogenicity of soots (Steiner, 1954; Kotin, 1956); e.g. (1) the presence of adequate solvent, (2) the adsorptive capacity of the adsorbent soot, (3) particle size which would determine penetration and settling out distal to the trachea.

With reference to the petrol exhaust soot, condition (1) is believed to be met to some extent due to the presence of the absorbed oil which was shown to dissolve aromatic hydrocarbons. The relatively low proportion of free carbon to the aromatic carcinogens would tend to minimize the adsorptive check (condition (2)). With regard to condition (3), Waller (1952), quoting many references, states that the exhaust particles of internal combustion engines " may be quite near to the range which undergo maximum retention in the lung ".

SUMMARY

1. An investigation of the aromatic polycyclic hydrocarbons occurring in samples of petrol exhaust, general atmospheric and diesel exhaust soots (the latter under one condition of engine operation only) was undertaken, and fluorescence and absorption spectra of some compounds, not hitherto recorded in the present connection, presented.

2. The soots were compared on the basis of the concentration of three commonly occurring hydrocarbons, anthracene, pyrene and 3,4-benzpyrene and petroleum-ether extractable material, and the possible biological significance of an associated oil in the case of petrol exhaust soot in particular, stressed.

3. A chromatographic procedure employed for the fractionation of the aromatic hydrocarbon mixtures is outlined.

We wish to thank Dr. P. R. Peacock, Director of Research, for helpful advice, and Mr. S. Breslin for photographic assistance. One of us (H. J.) is indebted to the Medical Research Council for a grant.

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COMPARISON OF THE CARCINOGENIC EFFECT OF X-IRRADIATION WITH RADIOACTIVE IODINE ON THE RAT'S THYROID

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Received for publication December 15, 1956

RATS injected with 30 μC radioactive iodine (I^{131}) and kept on methylthiouracil for the succeeding 15 months show adenomatous replacement of the thyroid gland and occasional thyroid carcinomas; unirradiated controls treated with methylthiouracil for 15 months develop a moderate number of adenomas but no carcinomas (Doniach, 1950 and 1953). The radiation dose, which is comparable to that used in the treatment of Graves' disease, varies extremely widely within the rat's thyroid gland. The following experiment is an attempt to find out whether radiation in the lower part of the dosage range, given before the goitrogenic treatment, is effective or not in initiating carcinogenesis. Dosage uniformity within the thyroid was obtained by irradiation with X rays. Comparison was made between the carcinogenic effect of 30 μC I^{131} with a single exposure of the thyroid to 1100 rads X rays. This dose of X rays was chosen because we had found previously in a biological assay that it was comparable with 30 μC I^{131} in its potency to inhibit the response of the rat's thyroid to a goitrogenic challenge given 4 months after the irradiation (Doniach and Logothetopoulos, 1955; Abbatt, Doniach, Flanders and Logothetopoulos, 1957).

MATERIAL AND METHODS

One hundred and sixty animals were used. They were black and white hooded male and female rats from a closed colony of the hooded Lister strain, fed on "Research" rat cubes with additional bread and greens. The 4-methyl-2-thiouracil (B.D.H.) was given as a saturated solution in the drinking tap water, made up once weekly by suspending 1 g. of the compound in each litre of water. The radioactive iodine, I^{131}, was injected intraperitoneally, carrier free as iodide in 1 ml. water. For X-irradiation deep anaesthesia was temporarily induced with ether vapour followed by intramuscular injection of 2·5 mg. Largactil (Chlorpromazine). The anaesthetized animals were immediately confined in a special holder and irradiated as described by Abbatt *et al.* (1957), the site of the thyroid having been marked with ink on the skin of the front of the neck. The X ray beam was defined by a perforated lead shield to a diameter of 1·3 cm. applied to the central surface of the neck immediately over the thyroid and portion of trachea between the 2 lobes. The physical factors were; 190 kV. X rays, 6 m.a., filtered by $\frac{1}{2}$ mm. Cu. and 1 mm. Al. The dose rate, taking the centre of the thyroid to be 8 mm. below the skin surface, was 150 rads/min. measured by means of a BD2 type condenser ionization chamber with the small air voume placed at this depth. 1100 rads were delivered to the thyroid of each rat. The irradiation was carried out by P. Howard Flanders of the Experimental Radiopathology Research Unit

of the Medical Research Council at Hammersmith Hospital. The animals averaged 3 months of age at the beginning of the experiment and were killed by coal gas 15 months later. The trachea and attached thyroid were fixed in Helly's fluid; the thyroid was then dissected off and weighed. When the thyroid was pathologically adherent to neighbouring structures, the actual adhesions to cervical connective-tissue, muscle or trachea were dissected off attached to the thyroid gland. Each thyroid was bisected in a horizontal plane through the isthmus and the two halves embedded in a single block. After trimming, a ribbon of about 12 serial sections was cut at 5 μ and mounted on 3 slides. One slide from each block was stained by haemalum and eosin, the spares were used for extra stains.

The rats were divided into 6 groups as follows: (1) Controls. (2) 30 μC I^{131}. (3) 1100 rads X rays. (4) Methylthiouracil in the drinking water for the duration of the experiment. (5) 30 μC I^{131} followed after 3 days by methylthiouracil until the end of the experiment. (6) 1100 rads X rays followed after 24 hours by methylthiouracil till the end of the experiment. The animals on methylthiouracil were given a rest from this toxic drug for 8 weeks during the 9th and 10th month.

Six months after the start of the experiment 3 males and 2 females taken at random from each of groups (1), (2) and (3) were put on to propylthiouracil in the drinking water, 6 mg./10 ml., and killed at the end of 12 days. Their thyroids were removed and weighed in order to confirm an equivalent inhibition of induced goitrogenesis in the I^{131} and X-irradiated animals.

RESULTS

The subsidiary test described above removed 15 rats from the original 160. A further 33 animals died and were discarded. Final histology was carried out on 112 rats, 66 males and 46 females.

Inhibition of goitrogenesis

The results, given in Table I, show a definite and more or less equivalent reduction in goitrogenic response in all the irradiated rats. The average weight of the control goitres was 46 mg., that of the I^{131} group 27 mg. and of the X-irradiated group 29 mg.

Main experiment

The findings are summarized in Table II–VII. The histological findings were the same as those described previously (Doniach, 1950 and 1953). No morphological

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EXPLANATION OF PLATE

FIG. 1.—Thyroid of female X ray rat 9B showing carcinomatous infiltration of the capsule. × 60.

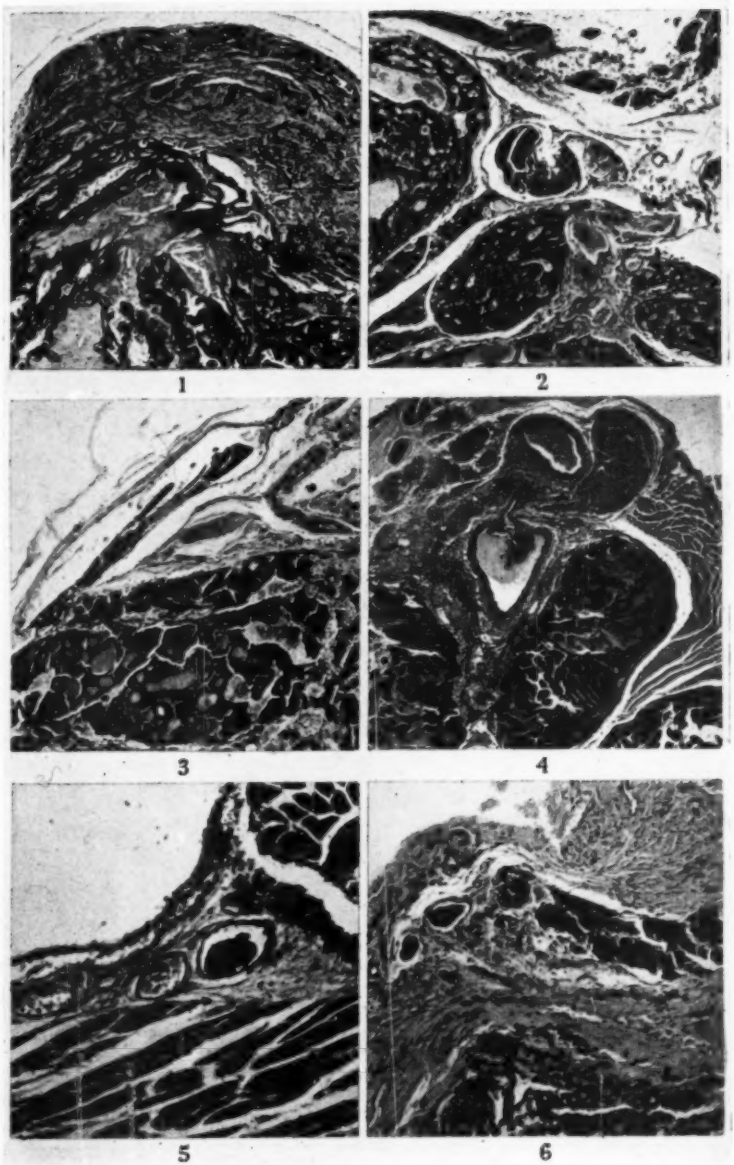
FIG. 2.—Thyroid of female I^{131} methylthiouracil rat 16C showing adenomatous replacement and carcinomatous permeation of a capsular vein. × 60.

FIG. 3.—Thyroid of male X ray methylthiouracil rat 5C showing adenomatous replacement and carcinomatous permeation of a capsular vein. × 60.

FIG. 4.—Perithyroid tissue of female X ray methylthiouracil rat 6B showing extension of adenomatous growth into cervical muscle and connective tissue. × 20.

FIG. 5.—Cervical muscle of female X ray methylthiouracil rat 6C showing permeation of an extrathyroidal vein with a thyroid carcinomatous acinus. × 120.

FIG. 6.—Thyroid of female X ray methylthiouracil rat 7A showing carcinomatous permeation of extracapsular veins. × 120.



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differences were found between the effects of I^{131} administration and X-irradiation. Adenomas indicate discrete nodules of thyroid tissue clearly separate and of a different morphology from surrounding parenchyma. Nodules smaller than 0·5 mm. in diameter and discrete foci of thyroid tissue which differed only slightly from surrounding parenchyma were regarded as border line tumours and were not included in the final assessment. Adenomatous replacement of one or both lobes, designated ++++ in the tables, refers to the presence of large and numerous adenomas associated with distortion and diminution of surrounding parenchyma and usually with gross fibrous thickening of the thyroid capsule. Carcinoma was diagnosed in all but one instance by the finding of tumour tissue lying within the lumen of capsular or extra-capsular veins. The exceptional carcinoma showed massive invasion of the capsule by dedifferentiated tumour cells. The findings are not quantitatively comparable with those of the author's previous similar experiments because this time the thyroids were not serially sectioned (Doniach, 1950) or cut at 6 levels (Doniach, 1953). This has reduced the number of adenomas counted but has not materially altered the quantitative findings in thyroids showing adenomatous replacement or malignant change.

There were 17 rats in the controls (Table II). Three of them showed small

TABLE I.—*Goitrogenic Response 6 months after Irradiation*

	Controls		30 μC I^{131}		1100 rad X rays	
Sex	Body weight (g.)	Thyroid weight (mg.)	Body weight (g.)	Thyroid weight (mg.)	Body weight (g.)	Thyroid weight (mg.)
M.	300	55	330	31	300	30
,,	340	44	360	32	320	28
,,	300	42	280	22	260	31
F.	240	48	230	24	210	25
,,	200	41	250	25	210	30

All rats were killed at the end of a 12-day course of propylthiouracil 6 mg./10 ml. in the drinking water.

TABLE II.—*Controls*

Rat	Sex	Body weight (g.)	Thyroid weight (mg.)	Micro-adenomas
23A	F.	220	23·0	+
23B	,,	215	13·0	—
23C	,,	195	25·1	—
23D	,,	230	28·1	—
23E	,,	205	20·1	—
24A	,,	225	18·0	+
24B	,,	225	22·6	—
24C	,,	235	29·8	—
25A	M.	295	24·6	—
25B	,,	295	28·4	—
25C	,,	335	33·5	—
25D	,,	350	33·7	+
25E	,,	345	32·9	—
26A	,,	325	22·5	—
26B	,,	325	32·4	—
26C	,,	335	27·9	—
26D	,,	290	25·9	—

Rats with the same number in the first column are from the same cage.

+ Represents the finding of scattered microadenomas.

collections of " solid " cellular follicles with occasional microfollicle development. There were no adenomas comparable with those found in the other groups.

Adenomas were present in 4 of the 22 rats of the I^{131} group (Table III) and in 4 of the 13 rats in the X-irradiated group (Table IV). One of the thyroids in the latter group weighed 160 mg. It showed adenomatous replacement of the left lobe with carcinomatous invasion of the capsule (Fig. 1). This thyroid was 5 to 10 times heavier than the other glands in both of these groups.

Of the 14 rats in the methylthiouracil group (Table V) 10 animals showed adenomas, the largest measuring 2·0 mm. across. There was no adenomatous replacement of any lobe.

TABLE III.—30 μC I^{131}

Rat	Sex	Body weight (g.)	Thyroid weight (mg.)	Number of adenomas	Diameter of largest adenoma (mm.)
10A	M.	305	14·5	1	0·5
10B	,,	350	27·8	1	0·5
10C	,,	350	19·9	—	—
10D	,,	390	20·7	—	—
11A	,,	405	19·0	1	0·5
11B	,,	270	17·5	—	—
11C	,,	370	37·8	2	1·5
11D	,,	280	20·3	—	—
12A	,,	240	14·0	—	—
12B	,,	345	22·6	—	—
12C	,,	345	21·7	—	—
12D	,,	355	20·3	—	—
13A	F.	230	20·3	—	—
13B	,,	265	16·2	—	—
13C	,,	270	18·3	—	—
13D	,,	190	13·7	—	—
17A	M.	385	25·0	—	—
17B	,,	375	26·2	—	—
19A	F.	220	15·8	—	—
19B	,,	215	16·8	—	—
19C	,,	260	17·5	—	—
19D	,,	235	20·9	—	—

TABLE IV.—1100 *rads X rays*

Rat	Sex	Body weight (g.)	Thyroid weight (mg.)	Number of adenomas	Diameter of largest adenoma (mm.)
1A	M.	290	21·5	1	0·5
1B	,,	370	33·4	—	—
2A	,,	335	29·0	—	—
3A	F.	210	14·5	—	—
3B	,,	245	21·1	—	—
3C	,,	230	26·3	—	—
3D	,,	265	22·9	—	—
4A	M.	345	26·2	1	1·0
4B	,,	325	17·3	—	—
4C	,,	360	28·1	2	0·5
9A	F.	255	15·3	—	—
9B	,,	245	160·0	++++ Ca.	4·5
9C	,,	250	28·8	—	—

++++ represents adenomatous replacement of one or both lobes.

Ca. represents malignancy.

There were 24 rats in the group given 30 μC I^{131} followed by methylthiouracil (Table VI). All except 1 showed adenomas, 16 showed adenomatous replacement of the thyroid and malignancy was diagnosed in 5 (Fig. 2). The heaviest thyroid was 385 mg. and the largest adenoma 5·0 mm. in diameter. All except 1 of the 22 rats given 1100 rads X-rays followed by methylthiouracil (Table VII)) showed adenomas. There was adenomatous replacement of the thyroid in 17 animals and evidence of maligancy in 7 (Fig. 3, 4, 5, 6). The heaviest gland weighed 566 mg. (Fig. 4) and the diameter of the largest adenoma was 5·0 mm.

TABLE V.—*Methylthiouracil*

Rat	Sex	Body weight (g.)	Thyroid weight (mg.)	Number of adenomas	Diameter of largest adenoma (mm.)
20A	F.	215	114	3	0·75
20B	"	135	50	—	—
20C	"	180	89	1	0·5
20D	"	255	266	2	1·0
20E	"	205	207	3	0·75
21A	M.	220	161	—	—
21B	"	240	245	2	2·0
21C	"	230	272	7	1·0
21D	"	210	165	2	0·75
22A	"	240	204	—	—
22B	"	215	102	—	—
22C	"	295	208	2	1·0
22D	"	305	298	4	1·5
22E	"	315	211	2	0·75

TABLE VI.—30 μC I^{131} *and Methylthiouracil*

Rat	Sex	Body weight (g.)	Thyroid weight (mg.)	Number of adenomas	Diameter of largest adenoma (mm.)
14A	M.	215	165	4	2·0
14B	"	205	91	12	1·0
14C	"	245	69	++++	1·5
14D	"	245	127	++++	3·5
15A	F.	215	180	++++ Ca.	2·0
15B	"	230	96	++++	2·5
15C	"	200	116	++++ Ca.	2·5
16A	"	165	35	4	1·0
16B	"	145	38	3	0·5
16C	"	200	229	++++ Ca.	3·5
16D	"	205	75	++++	1·5
18A	M.	270	141	++++	2·0
18B	"	235	360	++++	3·5
18C	"	245	245	++++ Ca.	4·0
18D	"	255	139	++++	2·5
31A	"	260	212	++++	4·0
31B	"	300	385	++++	5·0
32A	F.	215	39	—	—
32B	"	200	328	++++	4·5
32C	"	205	165	++++ Ca.	3·5
33A	M.	295	207	++++	2·0
33B	"	265	52	1	0·5
33C	"	260	143	4	1·0
33D	"	255	167	8	1·5

++++ and Ca. as in Table IV.

TABLE VII.—1100 *rads X rays and Methylthiouracil*

Rat	Sex	Body weight (g.)	Thyroid weight (mg.)	Number of adenomas	Diameter of largest adenoma (mm.)
5A	M.	245	67	++++	2·5
5B	,,	285	98	2	1·0
5C	,,	180	148	++++ Ca.	3·0
6A	F.	220	193	++++	3·0
6B	,,	200	566	++++ Ca.	4·0
6C	,,	195	193	++++ Ca.	4·5
7A	,,	200	276	++++ Ca.	3·0
7B	,,	185	132	++++	2·5
7C	,,	220	186	++++	2·0
8A	M.	230	238	++++	3·5
8B	,,	235	93	++++	2·5
8C	,,	210	236	++++	2·5
27A	,,	305	153	++++	3·5
27B	,,	255	72	1	1·0
27C	,,	255	172	++++ Ca.	4·0
27D	,,	275	115	++++	2·5
28A	,,	215	375	++++ Ca.	5·0
28B	,,	185	149	++++	3·5
28C	,,	230	53	5	1·5
28D	,,	255	35	3	0·5
29A	F.	175	138	++++ Ca.	3·0
29B	,,	320	121	—	—

++++ and Ca. as in Tables IV and VI.

DISCUSSION

The findings show a close parallel between the effect of 1100 rads X rays and 30 μC I^{131} in adenoma production in the thyroid and carcinoma production when followed by the administration of methylthiouracil for 15 months.

Due to variations between uptake and retention of iodine and distance from the gland periphery of thyroid follicles it is ony possible to express the dosage of radiation to the rat thyroid from administered radioactive iodine as a range. In our animals injected intraperitoneally with 30 μC I^{131} we regarded the range to lie between 2000 and 24,000 rads (Abbatt *et al.*, 1957). I thought (Doniach, 1953) that the carcinogenic activity of 30 μC I^{131} might have been initiated by *beta* radiation of the order of 5000 to 10,000 rads and promoted by the subsequent pituitary thyrotrophic hormone (T.S.H.) stimulation induced by prolonged thiouracil. The dosage of 5000 to 10,000 rads from *beta* rays has been shown to be carcinogenic to the skin of experimental animals (Raper, Henshaw and Snider, 1951 ; Glücksmann, 1951). When we found that 1100 rads X rays and 30 μC I^{131} were equivalent in their potency to inhibit hyperplasia of the thyroid (Doniach and Logothetopoulos, 1955) I considered that this dose of X-irradiation which lies well below 5000 rads might be non- or only very weakly carcinogenic. If so, X rays might be used for the treatment of Graves' disease with little risk of future carcinoma development. However, the results have proved otherwise and require further analysis.

It is now generally accepted that prolonged stimulation with T.S.H. alone leads eventually to carcinoma formation in the rat's thyroid (Bielschowsky, 1955 ; Axelrad and Leblond, 1955). The excess T.S.H. output may be produced by administrationof goitrogens (Purves and Griesbach, 1947) or by iodine deficiency (Axelrad and Leblond, 1955).

Carcinogenic action of T.S.H.

The development of tumours as a result of prolonged excessive stimulation of the thyroid by the pituitary is an example of experimental carcinogenesis without the agency of chemical or physical carcinogens in the usual sense. Though T.S.H. is a physiological secretion and its excessive output in gross iodine deficiency is a physiological compensatory phenomenon the resultant state of the thyroid is remarkably different from the normal resting gland. In the usual laboratory environment most of the cells of the adult rat thyroid last the animal's lifetime without renewal (Leblond and Walker, 1956). Goitrogen treatment leads to a tremendous wave of mitoses and a maintained marked cellular hypertrophy. With the passage of time multicentric adenomas appear in increasing numbers, grow and eventually show signs of malignancy. In T.S.H. carcinogenesis, therefore, it appears that an abnormally high rate of fission induced in normal cells has led to neoplasia. Analagous examples of tumour development are seen in ovarian auto-implants into the spleen of spayed mice, testicular auto-implants into the spleen of castrated rats, mammary glands of oestrogen-treated susceptible mice and possibly in the regenerating livers of cirrhotics.

Carcinogenic action of irradiation and summation with thyroid hyperplasia

Morphological evidence has been found in the thyroid (Maloof, Dobyns and Vickery, 1952; Doniach and Logothetopoulos, 1955) and pituitary (Goldberg and Chaikoff, 1950; Doniach, 1953) that there is a maintained increased output of T.S.H. following the administration of I^{131}. This appears to be a compensatory phenomenon which enables the damaged thyroid to put out a normal quantity of thyroid hormone at the expense of diminished hormone storage. Thus, the adenoma formation observed in the present experiment in the I^{131} and X-irradiated rat groups (2) and (3), may have resulted partly from the post irradiation increased output of T.S.H. However, after 30 μC I^{131} the average thyroid follicle cell height rises by only 16 per cent (Doniach and Logothetopoulos, 1955) whereas after methylthiouracil the cells are more than doubled in height. Since the increase in cell height is an index of the level of T.S.H. secretion, it follows that the T.S.H. level is far higher following methylthiouracil than after 30 μC I^{131}. It is possible that the increased numbers of adenomas in the methylthiouracil-treated rats, group (4), as compared with the irradiated ones, groups (2) and (3), reflects the greater output of T.S.H. On the other hand the increase in T.S.H. output in the irradiated animals appears to be comparatively slight and therefore unlikely to account by itself for adenoma production within 15 months. The tumours more probably result from the effects of summation of radiation with increased T.S.H. This is supported by the finding of one carcinoma in the X-irradiated group (3) in contrast to the absence of carcinomas in the methylthiouracil rats group (4), killed after 15 months, in this and in previous experiments (Doniach, 1950 and 1953) in spite of the considerably higher T.S.H. output in the latter.

The striking summation effect in carcinogenesis of radiation followed by methylthiouracil was noted previously (Doniach, 1950) and compared with the carcinogenic summation effect of acetylaminofluorene and goitrogens first demonstrated by Bielschowsky (1944). The added interest in the present experiment is that the effective dosage of radiation from I^{131} is equivalent to as little as 1100 rads X rays. This finding is of clinical interest since it suggests that X-irradiation

therapy in Graves' disease carries the same risk of future carcinoma development as I^{131} therapy. Also it fits the recent reports (Simpson, Hempelmann and Fuller, 1955 ; Clark, 1955) that carcinoma of the thyroid has developed in children whose thyroids were exposed to X-irradiation some years previously, in most cases th dose being well under 1000 rads. These children probably suffered a summation of direct irradiation damage to the thyroid with a secondary maintained rise in T.S.H.

In the present experiment we are comparing the effects of a dose of 190 kV. X rays delivered in 8 minutes with a dose of *beta* rays from I^{131} delivered at a falling rate over a few days. One cannot therefore assume that the effective carcinogenic dose in the I^{131} range was 1100 rads. One can postulate that it is likely to be well under 5000 rads, possibly 1500 to 2000 rads. In a previous experiment (Doniach, 1953), 5 μC I^{131} followed by methylthiouracil for 15 months led to an incidence of adenomatous replacement of the thyroid comparable with that following 30 μC I^{131}. However, tumour cells were not found in extracapsular veins in any rat and malignancy was not diagnosed. The dosage range from the 5 μC I^{131} was thought to lie between 380 and 2700 rads. This suggests that the effective carcinogenic radiation dose from I^{131} is above 2700 rads. However, the lack of development of overt malignancy at 15 months may have been related to the number of cells exposed to the carcinogenic range of radiation. The number might well have been much smaller in the 5 μC I^{131} group than in the 30 μC I^{131} group. The morphological changes were strikingly suggestive of incipient malignancy and overlapped those found in the 30 μC I^{131} group in which 5 examples of overt carcinoma were diagnosed in 20 animals. 30 μC I^{131} depresses the mitotic response to T.S.H. of most but not all cells of the thyroid 3 to 4 months onwards after irradiation (Doniach and Logothetopoulos, 1955). After 100 μC I^{131} neoplasia was considerably reduced, when presumably still fewer cells escaped post-irradiation inhibition of mitosis (Doniach, 1953).

Further examples of carcinogenic summation of radiation with other stimuli

Carcinomas of irradiated human skin arise on a basis of indolent ulcers which appear many years after the original irradiations (literature reviewed by Furth, 1954). Glücksmann (1951) has studied precisely the sequence of events in mouse skin exposed to about 8000 rads *beta* rays. The radiation leads to ulceration of the skin which heals and later breaks down and heals repeatedly with eventual development of malignancy in the exposed site. Malignancy was both initiated by the radiation and promoted by regeneration consequent to delayed radiation induced ischaemia. It is likely that the high dosage level of radiation was necessary not so much to initiate carcinogenesis but to induce a non-specific stimulus to tissue regeneration. Mottram (1938) obtained tumours of mouse skin by the summation of a non-carcinogenic course of benzypyrene painting (as judged by his controls) with a single exposure of 800 to 2500 rads *beta* rays. Similar results were obtained by Hamilton and Passonneau (1949) with the summation of *beta* rays and methylcholanthrene and by Boag and Glücksmann (1956) with *beta* rays and 1 : 2 : 5 : 6-dibenzanthracene or 9 : 10-dimethyl-1 : 2-benzanthracene. Lacassagne (1933) found that X-irradiation of inflammatory sites in the rabbit gave rise to connective tissue tumours. This was confirmed by Burrows, Mayneord and Roberts (1937) who obtained metastasizing sarcomas by giving 600 rads X rays to rabbits at the site of subcutaneous silica granulomas.

CONCLUSION

It appears that maintained tissue hyperplasia, not necessarily induced by a known carcinogen, may in certain circumstances lead to neoplasia. This tendency to neoplasia is heightened by a comparatively small dose of ionizing irradiation. With regard to the thyroid the non-specifically carcinogenic stimulus to hyperplasia is an excessive secretion of T.S.H. Daily small doses of thyroid hormone partly replace endogenous thyroid hormone secretion and thereby lower T.S.H. output. This treatment applied to patients whose thyroids have been irradiated should reduce the likelihood of subsequent thyroid neoplasia.

SUMMARY

The thyroids of 6 groups of rats, killed 15 months after the beginning of the experiment, were examined histologically for adenomas and carcinomas after the following different types of treatment: (1) Controls. (2) 30 μC I^{131}. (3) 1100 rads X rays to the thyroid. (4) Methylthiouracil for 15 months. (5) 30 μC I^{131} followed by methylthiouracil. (6) 1100 rads X rays to the thyroid followed by methylthiouracil. A few thyroids containing small adenomas were found in the I^{131} and 1100 rads X ray group. In addition, one of the X ray group showed a thyroid carcinoma. Small adenomas were present in most of the methylthiouracil group thyroids. Most of the thyroids of both the irradiated groups which were treated with subsequent methylthiouracil showed adenomatous replacement. In addition, 5 carcinomas were identified in the 24 rats given I^{131} followed by methylthiouracil. Seven carcinomas were identified in the 21 rats given 1100 rads X rays followed by methylthiouracil.

Thus, a single exposure of 1100 rads X rays proved equivalent to 30 μC I^{131} in its potency to initiate carcinogenesis in the thyroids of rats subsequently treated with methylthiouracil for 15 months. This finding is discussed in relation to the range of dosage to the thyroid of 2000 to 24,000 rads from 30 μC I^{131} and from the point of view of the carcinogenic summation of a comparatively small dose of radiation with maintained hyperplasia induced by prolonged excessive output of pituitary T.S.H.

I am grateful to Dr. P. Howard Flanders for carrying out the X-irradiation and for criticism of the manuscript, to Dr. J. H. Logothetopoulos for help in setting up the experiment, to Mr. J. G. Griffin and colleagues for the sections, to Mr. I. T. Hinton for the photomicrographs and to Mrs. D. Gearon for secretarial assistance.

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A QUANTITATIVE STUDY OF THE SYSTEMIC INITIATING ACTION OF URETHANE (ETHYL CARBAMATE) IN MOUSE SKIN CARCINOGENESIS

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Received for publication November 9, 1956

URETHANE has been shown to induce the initiating phase of skin carcinogenesis in mice, not only when applied locally (Graffi, Vlamynch, Hoffmann, and Schulz, 1953; Salaman and Roe, 1953; Roe and Salaman, 1954; Berenblum and Haran, 1955*b*), but also when administered by mouth (Haran and Berenblum, 1956). The fact that urethane (ethyl carbamate) has a relatively simple chemical structure, is water-soluble, is itself non-carcinogenic for skin epithelium in the mouse, and does not even elicit epithelial hyperplasia, or any other demonstrable evidence of skin irritation—so constant a feature of other initiating agents or skin carcinogens, renders it all the more attractive a tool for the study of the nature of initiating action.

Before one could effectively exploit urethane for this purpose, some quantitative data were needed about the conditions under which its systemic initiating action operated. The results of such a quantitative study, presented here, are concerned with (*a*) variations in total dose of urethane, given once only by mouth; (*b*) different numbers of urethane feedings, totalling the same overall dose; (*c*) different routes of administration of urethane, e.g. by mouth, intraperitoneally, subcutaneously, and topically applied to the skin; and (*d*) variations in the interval between the end of initiating action (urethane by mouth) and the commencement of promoting action (croton oil applied repeatedly to a small area of skin).

METHODS

The animals used in these experiments were male and female mice of the Swiss strain, bred in these laboratories by brother-sister mating for 16-18 generations. They were 2–3 months old at the start of the experiment, housed in an air-conditioned room at 21–23° C, and fed on *Purina Laboratory Chow*, and water *ad libitum*. The urethane was made up as a 5 per cent solution in distilled water, for oral, subcutaneous, and intraperitoneal administration, and as a 40 per cent solution in acetone, for skin application. The oral administration was by means of a polyethylene stomach tube, as previously described (Berenblum and Haran, 1955*a*). The promoting action with croton oil was standardized for the whole series of experiments described here, consisting of applications, with a glass rod, of a 5 per cent solution in liquid paraffin, limited to a small area (about 1·5 × 2 cm.) in the dorsal region of the back, the procedure repeated twice-weekly for 26 weeks.

The resulting skin tumours were charted at their first appearance, and fortnightly thereafter. Papillomas that regressed within 2 weeks of their first

appearance were not listed in the final records. Careful note was also taken at autopsy of the numbers of adenomas of the lungs in each animal.

RESULTS

1. *Effect of dose of urethane, administered orally, as initiating agent*

The effects of single feedings of urethane, containing 1, 4, 16, and 64 mg., respectively, followed in each case by twice-weekly applications of croton oil to the skin, are summarized in Table I. An increased response with increased dosage is clearly demonstrated in the form of (*a*) percentage of animals bearing papillomas, and (*b*) average number of papillomas per animal, and also of (*c*) percentage of mice bearing lung adenomas, and (*d*) the average number of lung adenomas per animal. On the other hand, the average latent period for papilloma production, though varying from group to group with the shortest latent period for the highest dose, showed no consistent correlation with dosage.

TABLE I.—*Effect of Dose of Urethane (Single Feeding), followed by Standard Croton Oil Treatment, on Tumour Induction*

Initiating agent	Promoting agent	Tumours of skin: Mice bearing papillomas/ survivors		Tumours of skin: Average number papillomas per mouse.	Tumours of skin: Average latent period* (weeks)	Tumours of lungs: Mice bearing adenomas/ survivors		Tumours of lungs: Average number adenomas per mouse
Urethane 64 mg.	Croton oil	24/24	100%	5·0±0·7	8	24/24	100%	7·0±0·9
„ 16 mg.	„	15/24	62·5%	1·2±0·3	14½	18/24	75%	1·6±0·2
„ 4 mg.	„	6/14	43%	0·4±0·2	20½	3/14	21%	0·3±0·2
„ 1 mg.	„	3/24	12·5%	0·25±0·1	16	4/24	17%	0·2±0·1
— (control)	„	2/20	10%	0·1±0·07	15½	1/20	5%	0·05±0·005

Urethane administered as a 5 per cent solution in distilled water by stomach tube.
Croton oil applied repeatedly to the skin (twice-weekly for 26 weeks), as a 5 per cent solution in liquid paraffin, by means of a glass rod.
Interval between initiating action and commencement of promoting action : 3 days.
* For first tumours.

While the lung tumour incidence, even in the group with the lowest dose of urethane (1 mg.), was significantly higher than the spontaneous incidence in the control group (without urethane), the difference *for skin tumours* between the group receiving only 1 mg. urethane and the control seems too small to be significant. The minimal effective dose of urethane *per os* for skin initiating action would, therefore, seem to lie somewhere between 1 and 4 mg.

2. *Different numbers of urethane feedings, totalling the same overall dose*

The total overall dose of urethane chosen for this experiment was the maximum tolerated dose, for our mice, *when given per os*, namely, 64 mg. This was administered as a single dose of 64 mg., as two doses of 32 mg., as 5 doses of 13 mg., and as 20 doses of 3·2 mg., respectively, the interval between the successive feedings being half a week. This treatment was followed, after a further interval of 3 days, by twice-weekly applications of croton oil for 26 weeks.

The results (Table II) are somewhat ambiguous, in that the first two groups (receiving one and two doses respectively) showed a high incidence of skin tumours

per animal and also of lung tumours, while the other two (receiving 5 and 20 doses) showed a relatively low incidence for both types of tumours; yet no consistent progression in response, in passing from 1 to 20 doses, was discernible.

TABLE II.—*Effect of Divided Doses of Urethane* per os, *followed by Standard Croton Oil Treatment on the Skin, on Tumour Induction*

Initiating agent	Promoting agent	Tumours of skin: Mice bearing papillomas/ survivors		Tumours of skin: Average number papillomas per mouse	Tumours of skin: Average latent period (weeks)	Tumours of lungs: Mice bearing adenomas/ survivors		Tumours of lungs: Average number adenomas per mouse
Urethane								
64 mg. × 1	Croton oil	24/24	100%	5·0±0·7	8	24/24	100%	7·0±0·9
32 mg. × 2	,,	37/37	100%	8·0±0·7	6	36/37	97%	9·0±0·5
13 mg. × 5	,,	17/20	85%	2·6±0·6	10½	13/20	65%	1·7±0·4
3·2 mg. × 20	,,	21/24	87·5%	2·2±0·4	11	20/24	83%	3·3±0·8

Details of method, as in Table I.

3. *Urethane action by oral, subcutaneous, and interperitoneal routes*

The maximum tolerated dose *per os* (64 mg.) was found to cause some deaths when given intraperitoneally. The somewhat smaller dose of 50 mg. was, therefore, chosen for both intraperitoneal and subcutaneous injections. In spite of this slight discrepancy in dosage, when compared with that used orally, it seems clear (Table III) that the dose of urethane, both for initiating action on skin and for complete carcinogenic action on the lung, is of the same order of magnitude for the three routes—orally, intraperitoneally, and subcutaneously.

TABLE III.—*Effect of Route of Administration of Urethane, followed by Standard Croton Oil Treatment on Skin, on Tumour Induction*

Initiating agent (urethane) (single dose)	Promoting agent	Tumours of skin: Mice bearing papillomas/ survivors		Tumours of skin: Average number papillomas per mouse	Tumours of skin: Average latent period (weeks)	Tumours of lungs: Mice bearing adenomas/ survivors		Tumours of lungs: Average number adenomas per mouse
64 mg. *per os*	Croton oil	24/24	100%	5·0±0·7	8	24/24	100%	7·0±0·9
50 mg. intraperitoneally	,,	28/28	100%	5·7±0·7	8	26/28	93%	3·0±0·4
50 mg. subcutaneously	,,	22/22	100%	4·3±0·2	10	22/22	100%	4·8±0·6

Details of method, as in Table I, except for route of administration, as indicated above.

4. *Comparisons with skin application (collar experiment)*

Strict quantitative comparisons between the action of urethane by the systemic routes and that resulting from direct skin application was impossible, because of the difficulty in determining the proportion of the applied urethane which is absorbed through the skin. There was, nevertheless, one important aspect of the problem that had to be examined, namely, whether the urethane applied to the skin surface did, in fact, penetrate at all, or whether its action

depended on the substance being licked off and swallowed, *thereafter acting systemically*.

To test this, a special plastic collar (Fig. 1) was designed, which would prevent the mouse licking the painted area of skin or the paws that scratched that area. [Preliminary tests, using a highly fluorescent hydrocarbon (3 : 4-benzpyrene) painted on the skin in mice with and without the collar, showed conclusively that the procedure was adequate for preventing the applied material finding its way to the system through the mouth. Fluorescence was readily observed in the contents of the stomach, soon after the skin painting, in mice without the collar, but no trace of fluorescence could be detected in those wearing it.]

Two groups of mice were treated identically, receiving 4 twice-weekly paintings of urethane to the skin, and later, repeated applications of croton oil according to the standard procedure, except that one group was made to wear the collar during the urethane treatment and for one week thereafter (by which time, all the urethane on the skin would either have been absorbed, or would have volatilized). Each mouse was kept in a separate cage during that period. The results (Table IV) do not substantiate the idea that urethane only acted by mouth, since both groups developed tumours of the skin and the lungs. However, the number of tumours per animal was higher in the group without the collar, thus indicating that some of the urethane, at least, normally reaches the systemic circulation by the intestinal route, through licking.

TABLE IV.—*Test for Route of Action of Urethane when Applied Locally to the Skin (Collar Experiment)*

Initiating agent (urethane applied to skin)	Promoting agent	Tumours of skin: Mice bearing papillomas/ survivors		Tumours of skin: Average number papillomas per mouse	Tumours of skin: Average latent period (weeks)	Tumours of lungs: Mice bearing adenomas/ survivors		Tumours of lungs: Average number adenomas per mouse
With collar	Croton oil	22/29	76%	1·0±0·3	14½	14/29	48%	0·6±0·2
Without collar	„	23/28	82%	1·7±0·3	10½	25/28	89%	2·0±0·3

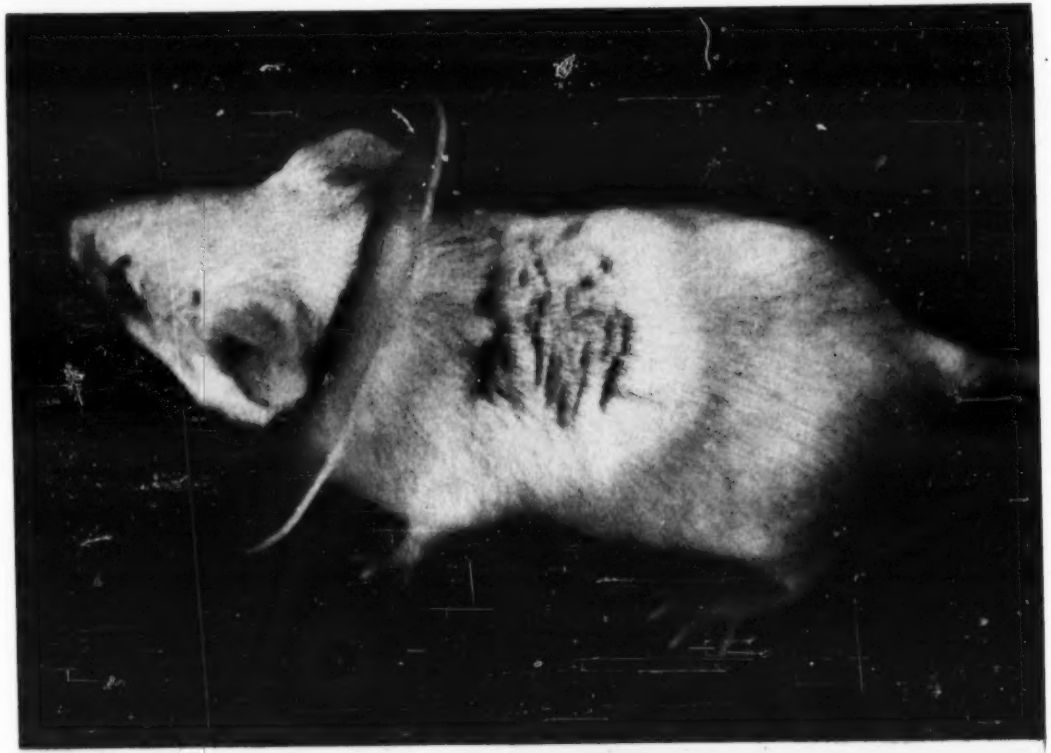
* Urethane, 40 per cent in acetone, applied twice-weekly for two weeks, to the skin.
Interval between last application of urethane and first application of croton oil : 7 days.
Croton oil treatment, and other details of method, as in Table I.

5. *Variations in interval between initiating and commencement of promoting action*

A single dose of 25 mg. of urethane was given by mouth, as initiator, and skin applications of croton oil, twice-weekly for 26 weeks, served as promotor, the interval between the two, in the different groups, ranging from 30 minutes to 56 days. The results (Table V) failed to show any striking difference in response either in skin tumour or lung tumour incidence, or in the number of tumours per animal, except for one case (56 days interval), in which the lung tumours per animal were significantly higher than in any of the other groups.

EXPLANATION OF PLATE

FIG. 1.—Mouse wearing plastic collar to prevent it licking the painted area of skin.



1

In a second series, 50 mg. of urethane, injected *subcutaneously*, was followed after 3 and 56 days, respectively, by the standard croton oil treatment on the skin. In this series (Table V), the number of skin papillomas per animal was somewhat higher in the case of the shorter than in that of the longer interval.

TABLE V.—*Influence of Length of Interval Between Initiating Action and Commencement of Promoting Action on Tumour Induction*

Initiating agent (urethane)	Interval	Tumours of skin: Mice bearing papillomas/ survivors	Tumours of skin: Average number papillomas per mouse	Tumours of skin: Average latent period (weeks)	Tumours of lungs: Mice bearing adenomas/ survivors	Tumours of lungs: Average number adenomas per mouse
25 mg. *per os*	½ hour	20/20 100%	3·1±0·4	9	17/20 85%	2·7±0·5
„ „	3 hours	18/20 90%	2·8±0·5	7	16/20 80%	2·7±0·5
„ „	5 „	18/19 95%	3·0±0·4	9	15/19 80%	1·7±0·3
„ „	1 day	16/19 84%	1·5±0·3	11	13/19 68%	2·2±0·6
„ „	2 days	18/20 90%	1·5±0·5	11	12/20 60%	1·5±0·4
„ „	3 „	33/40 82·5%	1·6±0·2	11	34/40 85%	2·7±0·3
„ „	7 „	36/38 94%	2·4±0·3	11	28/38 74%	2·3±0·4
„ „	14 „	18/20 90%	2·4±0·4	11	17/20 85%	2·4±0·3
„ „	56 „	34/37 92%	2·0±0·3	9	35/37 94%	4·8±0·6
50 mg. s.c.	3 „	22/22 100%	4·3±0·2	10	22/22 100%	4·8±0·6
„ „	56 „	16/20 80%	2·3±0·4	10	18/20 90%	3·7±0·65

Details of method, as in Table I, except for injection by the subcutaneous route in the last two experiments.

6. *Influence of sex*

Since both male and female mice were used in these experiments, some information about the influence of sex on the present method of carcinogenesis (for this particular strain of mice) might be obtained. Separate data for the two sexes are not shown in Tables I–V, since the number of animals per group would have been too small for statistical evaluation. Since a sex difference nevertheless was suspected, on the grounds that the small observed differences were consistently in the same direction, namely, towards a higher incidence for females, the figures in Table V were pooled, and analysed according to sex, providing the following results :

Average number papillomas per mouse : 2·7 ± 0·2 for females ; 1·7 ± 0·2 for males ;

Average number of lung tumours per mouse : 3·5 ± 0·3 for females ; 1·7 ± 0·2 for males.

These values are based on 96 females and 97 males.

7. *Possible correlation between the induced tumours of the skin and lungs*

In view of the double action of urethane—initiating action on the skin and complete carcinogenic action on the lungs, and since, under the conditions of the present experiments, the tumour yield in either tissue was often below 100 per cent, it was interesting to determine whether there was any correlation between responsiveness to one action and the other. No such correlation could be detected.

DISCUSSION

The two-stage mechanism of skin carcinogenesis postulates an *initiating phase*, representing an irreversible transformation of some normal into "latent" or "dormant" tumour cells, followed by a promoting phase, responsible for their conversion into a growing tumour. This concept arose from certain experiments in rabbits, in which tar-induced skin papillomas that had regressed, were made to reappear by further treatment with a variety of stimuli (Rous and Kidd, 1941; MacKenzie and Rous, 1941), and from subsequent experiments in mice, in which applications of a carcinogen, insufficient to induce tumours, followed by croton oil treatment, resulted in the appearance of papillomas (Berenblum, 1941; Mottram, 1944; Berenblum and Shubik, 1947; etc.). In both types of experiment, the agent used for initiating action was a complete carcinogen, the promoting component being restricted by limiting the period of treatment (usually to a single application, in the case of the mouse experiment).

Initiating action by the application of urethane to the mouse's skin (Graffi, Vlamynch, Hoffmann, and Schulz, 1953; Salaman and Roe, 1953; Roe and Salaman, 1954; Berenblum and Haran, 1955*b*) provided a refinement in the technique, in that this compound is non-carcinogenic for mouse's skin, even when applied twice-weekly for 43 weeks (Berenblum and Haran, 1955*b*), and also for reasons already referred to in the introduction of this paper. The fact that urethane also acted as an initiating agent for the mouse's skin *when given by mouth* (Haran and Berenblum, 1956), provided certain new approaches to the study of the mechanism of skin carcinogenesis, in that many of the side reactions, resulting from *local* application of an initiating agent, were thereby eliminated.

The first stage of such a study must necessarily be concerned with comparisons between the effectiveness of different routes of administration for systemic action, and with the verification as to whether systemic initiating action was analogous to that arising from local action on the skin. These investigations, reported here, were carried out quantitatively, for additional information.

The fact that urethane given orally, intraperitoneally, and subcutaneously, acted more or less equally effectively for initiating action on the skin (Experiment 3), eliminated the possibility that chemical changes in the gut, and absorption through the intestinal mucosa, modified the effect in any way. The idea that the urethane, applied to the skin, might act indirectly, by being first swallowed through licking (Experiment 4)—an attractive possibility, in view of the absence of any evidence of local irritation—was found to be untenable. Thus, whatever the route by which the urethane enters the system (even by absorption through the skin), it is capable of acting both as an initiator for skin carcinogenesis and as a complete carcinogen for the lung.

The fact that the tumour yield in the skin, resulting from a single feeding of urethane followed by repeated local applications of croton oil to the skin, was determined by the dose of urethane, while the average latent period was not (Experiment 1), is in agreement with the earlier results involving local application of polycyclic aromatic hydrocarbon carcinogens for initiating action (Berenblum and Shubik, 1947), and fails to substantiate some recent criticisms regarding the quantitative relationships of the two-stage mechanism of carcinogenesis. These dealt with two aspects of the problem—(*a*) concerning the quantitative correlation

between dose of initiator and yield of tumours, and (*b*) concerning the lack of correlation between dose of initiator and average latent period.

In a series of papers by Roe (1956*a*, 1956*b*) and Salaman and Roe (1956*a*, 1956*b*), experiments are described in which prolongation of the croton oil treatment in the standard experiment (with local application of a hydrocarbon carcinogen as initiator) led to a progressive increase in tumour yield, which seemed contrary to the levelling-off of the curves in the original experiments of Berenblum and Shubik. Since both the carcinogen (used as initiator) and the croton oil (used as promotor) may elicit some " background " carcinogenic action, it might be expected that, after very prolonged treatment, additional tumours would appear as a consequence of these complicating factors. The ideal set-up would be to use a " pure " initiator and a " pure " promotor for testing the hypothesis. We do not yet possess a pure promotor (i.e. *completely* lacking in initiating action), though a pure initiator, in the form of urethane, is now available. The results with urethane, described here, are consistent with the original hypothesis that the *number* of tumours is a function of initiating action while the *latent period* is a function of promoting action. Regarding the latter, the latent period (Experiment 1) varied from group to group, without a consitent correlation with the dose of initiator (as claimed by Klein, 1956).

A number of investigators (Graffi, 1953 ; Druckrey, 1954 ; Roe and Salaman, 1954 ; Klein, 1956) obtained a quantitatively cumulative effect when the same amount of initiator is divided into separate doses ; while some (e.g. Saffiotti and Shubik, 1956) found the small, repeated doses actually more effective than the large, single dose. The present results with urethane given orally (Experiment 2) also suggest a cumulative effect, though not quantitatively. No explanation is available to account for this, though the conditions of the experiment are more complicated than might at first appear, since the dividing up of the dose into several small doses also involves the spreading out of the initiating action over a longer period.

SUMMARY

The previous observation that urethane, administered by mouth, acted as an initiating agent for skin carcinogenesis in the mouse (i.e. rendering the skin responsive to subsequent promoting action by locally applied croton oil, with the development of papillomas) was confirmed. The intraperitoneal and subcutaneous routes were found to be about equally effective.

The yield of skin papillomas, resulting from the initiating action of orally administered urethane, followed by repeated skin applications of croton oil, was determined by the dose of urethane adminstered (tested over a range of 1–64 mg.), while the average latent period was not related to dose. This result is in keeping with the earlier findings of Berenblum and Shubik (1947), using 9 : 10-dimethyl-1 : 2-benzanthracene, applied locally, as initiator.

Varying the interval between the initiating action of urethane (by mouth) and the commencement of croton oil applications—tested over a range of 30 minutes to 56 days—did not significantly affect the tumour yield in the skin.

By means of a special " collar " experiment, which prevented the mice from licking the skin, it was possible to show that locally applied urethane acted through the skin rather than by being swallowed and absorbed through the intestine

(though a slightly higher tumour yield was observed in mice that were allowed to lick the skin).

The results are discussed in relation to other recent work on the " two-stage mechanism " of skin carcinogenesis.

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THE INDUCTION OF THE INITIATING PHASE OF SKIN CARCINOGENESIS IN THE MOUSE BY ORAL ADMINISTRATION OF 9 : 10 - DIMETHYL - 1 : 2 - BENZANTHRACENE, 20 - METHYLCHOLANTHRENE, 3 : 4 - BENZPYRENE, AND 1 : 2 : 5 : 6 - DIBENZANTHRACENE

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Received for publication November 9, 1956

THE initiating phase of skin carcinogenesis in the mouse can be induced not only when the agent is applied locally but also when administered systemically, as has been demonstrated with 9 : 10-dimethyl-1 : 2-benzanthracene (Graffi, Scharsach, and Heyer, 1955; Haran and Berenblum, 1956) and with urethane (Haran and Berenblum, 1956; Berenblum and Haran-Ghera, 1957). [2-Acetylaminofluorene, which is not an initiator for skin carcinogenesis when applied locally (Price, 1947; Salaman and Roe, 1953), does have that action when given by mouth (Ritchie and Saffiotti, 1955).]

Since initiating action by *local* application can also be induced with other carcinogenic compounds, such as 3 : 4-benzpyrene (Berenblum, 1941; Mottram, 1944; Friedewald and Rous, 1944*a*), 20-methylcholanthrene (Kline and Rusch, 1944; Friedewald and Rous, 1944*b*; Berenblum and Shubik, 1947), and 1 : 2 : 5 : 6-dibenzanthracene (Berenblum and Shubik, 1947), it was thought desirable to test also these systemically (by mouth), and to compare their potencies, under these conditions, with that of 9 : 10-dimethyl-1 : 2-benzanthracene.

METHOD

The animals used in these experiments were female Swiss mice, bred in these laboratories by brother-sister mating for 16–18 generations. They were 2–3 months old at the start of the experiment, kept in plastic cages, 8–10 mice per cage, and housed in an air-conditioned room at 21–23° C. They were fed on *Purina Laboratory Chow*, and water *ad libitum*.

For initiating action, the mice were given a single feeding, by stomach tube, of 0·3 ml. of a 1·0 per cent solution, in polyethylene glycol-400, of the 4 compounds, 9 : 10-dimethyl-1 : 2-benzanthracene (DMBA), 20-methylcholanthrene (MCA), 3 : 4-benzpyrene (BP), and 1 : 2 : 5 : 6-dibenzanthracene (DBA), respectively. For promoting action, a 5 per cent solution of croton oil in medicinal liquid paraffin was applied, with a glass rod, to an area of skin about 2 × 2 cm. in the dorsal region, the hair of the parts being previously clipped with fine scissors. This treatment was repeated twice-weekly for 32 weeks.

The resulting skin tumours (papillomas) were charted at their first appearance, and fortnightly thereafter. Papillomas that regressed within 2 weeks of their first appearance were not listed in the final records. The average latent periods were based on the times of appearance of the *first* tumours per animal, as on

previous occasions. They represent, therefore, average latent period with respect to *animal* response, rather than to the cell population under treatment.

RESULTS

The tumour yields, the average numbers of tumours per animal, and the average latent periods, are summarized in Table I. The corresponding values *at the end of the 20th week of croton oil treatment* are summarized in Table II (for reasons to be discussed below).

TABLE I.—*Development of Tumours of the Skin in Mice Receiving Various Hydrocarbons by Mouth, followed by Repeated Skin Applications of Croton Oil*

Initiation*	Promotion	Mice bearing papillomas/survivors	Average number papillomas per animal	Average latent period (weeks)
DMBA	Croton oil × 64	24/26 92%	8±1·5	9
MCA	" " " "	20/25 80%	3±0·4	16
BP	" " " "	14/18 78%	0·8±0·2	23
DBA	" " " "	12/23 52%	0·8±0·2	20
— (control)	" " " "	2/21 10%	0·09±0·06	29

* 3 mg. in polyethylene glycol-400—single dose by mouth.

TABLE II.—*As in Table I : Results after only 20 Weeks of Croton Oil Treatment*

Initiation*	Promotion	Mice bearing papillomas/survivors	Average number papillomas per animal	Average latent period (weeks)
DMBA	Croton oil × 40	24/26 92%	8·2±1·5	9
MCA	" " " "	14/25 56%	1·1±0·3	12
BP	" " " "	4/19 21%	0·2±0·09	14½
DBA	" " " "	7/24 29%	0·3±0·1	14
— (control)	" " " "	0/21 0%	—	—

* As in Table I.

The results show that while all four compounds, administered by mouth, induce the initiating phase of skin carcinogenesis in the mouse, DMBA is by far the most potent for this action.

DISCUSSION

The fact that all the four compounds tested (DMBA, MCA, BP, and DBA) can induce the initiating phase of skin carcinogenesis in the mouse when administered orally, suggests that systemic initiating action is not a freak phenomenon on the part of some unusual compound, but represents a fundamental aspect of skin carcinogenesis. If, as seems likely from tangential evidence (Berenblum, 1956), initiating action involves a prior metabolic conversion of the compound to some metabolite, attractive possibilities are opened up for the future study of the mechanism of carcinogenesis in biochemical terms.

With regard to the actual values obtained in these experiments, DMBA was found to be by far the most potent of the four compounds for initiating action by mouth, while BP and DBA were found to be relatively weak.

The variations in average latent periods (Table I) call for some comment, since no such variations were observed when these compounds were tested by local application (Berenblum and Shubik, 1947). In the latter experiments, the

croton oil treatment was only continued for 20 weeks. The corresponding data, at the 20th week, in the present experiment, also indicate a diminution in the differences in latent periods (Table II). As already suggested by us in another connection (Berenblum and Haran-Ghera, 1957), the differences in latent periods *in prolonged experiments* may be partly explained by " background " carcinogenesis by the croton oil itself. A further factor, to account for this, is connected with the fact that the values for latent periods refer to the times of appearance of the *first* tumours only per mouse, which, in the case of a potent initiator, would be likely to show less spread in the incidence curve than with a weak initiator, and this would be accentuated in long-term experiments. A single tumour appearing at the 32nd week, in a group in which the other tumours are few and appear early, would cause the *average* latent period for the whole group to be exaggeratedly delayed.

Thus, the conflict between the original thesis of Berenblum and Shubik (1947) that on the basis of the two-stage hypothesis of skin carcinogenesis, the tumour incidence is dependent on initiating action and the latent period only on promoting action, and the recent results of Roe (1956*a*, 1956*b*), Salaman and Roe (1956*a* 1956*b*), and Klein (1956) that in prolonged experiments, the initiator does appear to affect the latent period, could only be resolved (*a*) by finding a promoting agent without " background " carcinogenic action, and (*b*) by more accurate mathematical analysis of data from large-scale experiments, preferably based on latent periods that are derived from all the induced tumours of the skin, and not merely from the first tumour per animal.

SUMMARY

9 : 10-dimethyl-1 : 2-benzanthracene, 20-methylcholanthrene, 3 : 4-benzpyrene and 1 : 2 : 5 : 6-dibenzanthracene, were found to act as initiators for skin carcinogenesis in the mouse when administered by mouth.

Of these four compounds, 9 : 10-dimethyl-1 : 2-benzanthracene was the most potent for this action.

This work was supported in part by a grant from the Joseph and Helen Yeamans Levy Foundation, to whom the authors wish to express their indebtedness.

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A STUDY OF THE VASCULAR DISSEMINATION OF TUMOR CELLS IN CORTISONE-TREATED MICE

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Received for publication August 20, 1956

It has been reported that cortisone increases metastatic spread in mice bearing the following transplanted and induced tumors: mammary adenocarcinomas K7 (Agosin *et al.*, 1952), DBA (Baserga and Shubik, 1954), K9, Sa and Sb (unpublished work of Vergara *et al.*), bladder carcinoma T150 (Baserga and Shubik, 1955), methylcholanthrene-induced squamous cell carcinoma and methylcholanthrene-induced sarcoma (Baserga and Shubik, 1954).

The phenomenon, however, is not general and the following mouse transplantable tumors do not seem to be affected by treatment with cortisone: mammary adenocarcinomas Acc. 635 (Sparks *et al.*, 1955), Eo771 and C3HBA (unpublished observations of Gasic and Gasic), Sarcoma 1 (Kaliss, Borges, and Day, 1954) and Sarcoma 180, carcinogen-induced fibrosarcoma GL46, and DBA melanoma (unpublished work of Gasic and Gasic).

The opposite phenomenon has even been reported, namely that cortisone decreases the metastatic spread of a mammary adenocarcinoma which originated in a Z mouse (Martinez and Bittner, 1955).

In those cases in which cortisone enhances the spread, it has been shown that at least one of the actions of the hormone is concerned with the implantation and growth of the tumor emboli, following their release into the circulation (Pomeroy, 1954; Baserga and Shubik, 1955; Goldie *et al.*, 1955; unpublished results of H. Maturana and of J. Vergara *et al.*). However, no attempt has been made to investigate, in a direct way, whether cortisone may also influence the release of tumor emboli from the primary tumor. To study this possibility, the presence of stray neoplastic cells has been compared in cortisone-treated and untreated tumor-bearing animals.

MATERIALS AND METHODS

Three transplantable mouse tumors were used: the bladder carcinoma T150 (Baserga and Saffiotti, 1955) which can grow progressively in C57BL, C57BR, DBA/1, DBA/2 and C3H, the carcinogen-induced fibrosarcoma GL46 (Baserga and Baum, 1955) and Sarcoma 180 (Greene and Murphy, 1954). They were inoculated subcutaneously as cellular suspensions, into the left flanks of homologous hosts, each receiving $\frac{1}{2}$ or $\frac{1}{5}$ of a standard dose, contained in 0·20 ml. of a suspension prepared with one part of the tumor, finely minced with scissors, and two parts of saline.

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At variable intervals after transplantation, both cortisone-treated and untreated animals were bled from the right heart, under ether anaesthesia. To avoid puncturing the lung with the needle, the bleeding was made with the thorax opened. 0·50 or 0·75 ml. of blood was obtained and inoculated immediately into the peritoneal cavity of susceptible hosts. These recipients were allowed to live until natural death or were killed at variable intervals after blood inoculation. Both donors and the recipients were autopsied. In the former the weight of the primary tumor was determined and the number of lung metastases were counted. In the latter the presence of peritoneal growth and hemorrhagic ascites was ascertained.

Cortisone was given at variable periods after tumor inoculation, each animal receiving six or ten daily injections of 0·75 mg. of cortisone acetate (" Cortone ", Merck and Co.) intraperitoneally. To avoid infections in this group, the cortisone suspension was mixed with streptomycin and oxytetracycline, dissolved in isotonic aqueous KCl. Each mouse received 1 mg. of each antibiotic. The same amount of these substances, but without cortisone, was given to the control group.

EXPERIMENTAL

(a) *Carcinoma T150 into C57BL/6 mice*

One-eighth of the standard dose was transplanted into 2-month-old male and female mice in Experiments 1 and 2, and into 3·5-month-old male and female mice in Experiment 3. The first experiment consisted only of untreated mice whereas in Experiments 2 and 3 there were both cortisone-treated and untreated animals. These latter groups received ten injections of cortisone, which were started 10 and 14 days after tumor implantation, respectively. The bleeding was performed on the fifteenth day after transplantation in Experiment 1, on the twentieth day in Experiment 2, and on the twenty-sixth day in Experiment 3. The blood obtained was inoculated into 1 month-old C57BL/6 mice, generally of the same sex as the donors.

The results (Table I) are as follows :

TABLE I.—*Number of Recipients with Peritoneal Growth. Primary Tumor and Metastases in the Blood Donors*

	Blood donors						Recipients		
			Mice with metastases				Mice with peritoneal growth		
Exper. no.	Cortisone-treated	Average primary tumor wt. (g)	Rate	%	Av. number metastases per mouse	Time of bleeding (days after inoculation)	Rate	%	Av. sv. after inocul. (days)
1*	No	2·1	15/23	65	4·0	15	16/22	72	29
2*	No	2·1	15/19	79	7·0	20	17/19	89	26
	Yes	1·8	18/21	86	13·1	20	17/21	80	25
3*	No	2·8	34/37	92	11·0	26	27/33	82	24
	Yes	2·6	34/35	98	32·0	26	23/31	74	22

* Carcinoma T150.

Experiment 1.—(1) *Donors :* Lung metastases were observed in 15 of the 23 animals (65 per cent), the average number per mouse being 4. (2) *Recipients :* 16 out of 22 mice had peritoneal growth and hemorrhagic ascites (72 per cent) and lived 29 days following inoculation.

Experiment 2.—(2) *Donors:* Lung metastases were present in 15 of the 19 controls (79 per cent) and in 18 of the 21 cortisone-treated mice (86 per cent). The number of metastases per mouse was 7·1 and 13·1 respectively and corresponding figures for the primary tumor weights were 2·1 and 1·8 g. (2) *Recipients:* The survival of mice receiving blood from untreated donors was 26 days as compared with 25 days in the case of recipients of blood from treated donors. Malignant peritoneal growth with hemorrhagic ascites was observed in 17 of the 19 animals in the first group (89 per cent) and in 17 of the 21 in the second group (80 per cent).

Experiment 3.—(1) *Donors:* Thirty-four controls out of 37 (92 per cent) and 34 cortisone-treated mice out of 35 (98 per cent) showed lung metastases, with an average number of 11 and 32 respectively. The average weight of the primary tumor was 2·8 g. in the first and 2·6 g. in the second group. (2) *Recipients:* peritoneal growth with hemorrhagic ascites was seen in 27 of 33 animals (82 per cent) that received blood from untreated donors and in 23 of 31 mice (74 per cent) inoculated with blood from the treated donors. The survival time was respectively 24 and 22 days.

(b) *Fibrosarcoma GL46 into DBA/2 mice*

One-half the standard dose was inoculated into 2-month-old mice. They were only females in Experiment 4 and of both sexes in Experiment 5. Half the animals of Experiment 5 received ten injections of cortisone from the fifth day onward. Animals of the second group (Experiment 5) received no additional treatment. Female mice, 1 month old, acted as recipients of the blood drawn from Experiment 4 and Experiment 5, 15 and 16 days after tumor implantation. These mice were in turn sacrificed 60 and 53 days after blood inoculation.

The following results are recorded in Table II :

TABLE II.—*Number of Recipients with Peritoneal Growth. Primary Tumor and Metastases in the Blood Donors*

		Blood donors			Recipients	
Exper. no.	Cortisone treated	Average primary tumor wt. (g.)	Mice with metastases (Rate)	Time of bleeding (day after inoculation)	Mice with peritoneal growths (rate)	Av. Sv. after inoculation (days)
4*	No	1·7	0/21	15	0/21	60
5*	No	2·6	0/15	16	0/15	53
	Yes	1·8	0/14	16	0/14	53
6†	No	0·9	0/23	10	0/23	58
	Yes	0·9	0/24	10	0/22	58

* GL46—a transplantable fibrosarcoma.
† Sarcoma 180.

Experiment 4.—The average weight of the primary tumor in the donors was 1·7 g. No metastases were observed in 21 animals, nor were malignant peritoneal growths present in the 21 recipients of the blood from the untreated donors.

Experiment 5.—No metastases were evident in the 15 untreated or 14 cortisone-treated donors, nor was there peritoneal growth in any of the mice that received

blood from these donors. Their primary tumor weights were 2·6 g. in the untreated and 1·8 g. in the treated mice.

(c) *Sarcoma* 180 *into Swiss mice*

Two-month-old female Swiss mice received $\frac{1}{8}$ of the standard dose of the tumor. Six injections of cortisone were given to half of them, starting 5 days after transplantation. Ten days after tumor implantation these animals were killed and 0·5 ml. samples of their blood were inoculated into Swiss female mice, 1 month old. These recipients were killed 58 days later.

Results (Experiment 6 in Table II).—The weight of the primary tumor in both groups of donors averaged 0·9 g. No metastases were seen in any of these animals ; none of the 23 recipients of blood from the untreated donors nor of the 22 inoculated with blood from the cortisone-treated donors had peritoneal growths.

DISCUSSION

To study the emboli release from the primary tumor, the ideal method would be to draw the venous blood from the tumor itself and to collect it for as long a period as possible. As this is not possible in mice, it was decided to collect venous blood from the right heart, in an effort to get the maximum amount of circulating fluid. However, the amount of blood coming from the tumor must be small and presumably contained few malignant emboli.

The fact that recipients of blood from tumor-bearing donors show peritoneal growth, indicates, without any reasonable doubt, that tumor cells were present in the circulating blood. Though the absence of malignancy in the recipients does not exclude the presence of a few circulating tumor cells in the donors*, from a practical point of view, such lack of tumor growth will be assumed to indicate absence of circulating emboli in the donors. With this reservation, certain comments can be made about the experimental results reported.

In untreated tumor-bearing mice there seems to be a correlation between the presence of circulating emboli and the spontaneous capacity of the neoplasm for producing metastases. When tumor cells were detected in the circulation, secondary tumors were also detected in the lung. On the contrary, when the emboli were absent, no metastases were noted. Nevertheless, these results do not eliminate the possibility that lack of metastases may be associated with the presence of tumor emboli in the circulation in amounts which might be revealed by more subtle methods.

Cortisone induced a positive metastatic effect only when the host was inoculated with a malignant growth which, like *T*150, has the spontaneous capacity for delivering tumor emboli into the circulation. No such result was encountered with GL46 and Sarcoma 180, which under natural conditions do not release emboli into the circulation, at least during the survival time of the study. However, by using a bio-assay technique, Goldie *et al.* (1955) were able to detect the existence of tumor cells in the liver, lung and brain in untreated CFW mice, 6 days after transplantation of Sarcoma 180, but in this case the tumor was inoculated into the peritoneal cavity of the donors.

* It is known that the peritoneal take of a neoplasm may require in certain cases more than 50 cells (Sugiura, 1953) or 1000 (unpublished work of Barahona *et al.*).

It appears from these experiments that cortisone has no effect on circulating tumor emboli. When these are naturally absent, as in Sarcoma 180 and GL46, cortisone does not make them appear. When, as with T150 circulating emboli occur naturally, cortisone does not appear to modify their number.

In summary, it may be said that no evidence was found in this experimental work supporting participation of cortisone in the phenomenon of the emboli release from the primary tumor. This conclusion is also supported by other experiments of the authors (unpublished) in which the spontaneous release of emboli from tumor T150 is not correlated with an increase of the adrenal activity, measured by the blood eosinophilia and by the thymus and adrenal weights, before and after metastases have appeared.

SUMMARY

The occurrence of circulating neoplastic cells was studied in cortisone-treated and in untreated tumor-bearing mice which received isografts of three different neoplasms. It was found that the hormonal treatment did not modify the pre-existing number of circulating emboli. In addition, it was observed that the enhancing effect of cortisone on secondary tumors was only present in animals inoculated with a tumor which has the natural capacity of delivering malignant emboli into the circulation.

The authors are greatly indebted to Dr. Philippe Shubik and Dr. Jean Sicé for their suggestions, criticism and correction of the manuscript.

Thanks are due for technical help to Mrs. Joline Ozane and Mrs. Mayme Spencer.

This work was supported by a research grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

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OBSERVATIONS ON THE PATHOGENESIS OF SARCOMA 37 MOUSE ASCITES TUMOUR

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Received for publication January 7, 1957

TRANSPLANTABLE mouse ascites tumours provide a useful experimental tool for the application of quantitative methods to the study of cancer. However it is important to be aware of the conditions under which cells multiply in such tumours and of possible differences existing between them and, for example, "solid" subcutaneous tumours.

It has been suggested that the cells of ascites tumours multiply while freely suspended in peritoneal fluid. Another proposition would be that inoculated cells multiply within tissues in microscopic foci to produce daughter cells that are liberated from the foci to provide an increasing number in the peritoneal fluid. If the first hypothesis is correct then cells in suspension in ascitic fluid are actively metabolizing and dividing. Whereas, if the second hypothesis is true the cells in ascitic fluid may not be in a state of active metabolism and division; if this is so, the chemical and other properties of suspended cells may be different from those of cells in actively growing tumour tissue. It is clear then that, before drawing far reaching conclusions from experiments on ascites tumours, the metabolic condition of the constituent cells should be known.

Goldie and Felix (1951) concluded that the cells of the ascites form of Sarcoma 37 divided while in suspension in peritoneal fluid because they could detect an increase in number of cells in the fluid before vascularization could have taken place. Klein (1951) investigating the same tumour came to the same conclusion because he detected a similar increase in tumour cells in mice in which he could find no histological evidence of tumourous infiltration of intra-abdominal tissues. Therefore it is thought important to present the following contrary observation that growth of Sarcoma 37 ascites tumours takes place in tissue before an increase of cell number occurs in peritoneal fluid. In addition no entirely satisfactory explanation for the mechanism of accumulation of ascitic fluid following the intraperitoneal inoculation of ascites tumour cells has been presented. Observations providing some indication of how ascitic fluid accumulates under these conditions will be described.

Klein's (1951) definition of ascites tumours is such that it assumes multiplication of freely suspended cells. For this communication his definition is unsuitable. Transplantable mouse ascites tumours will be regarded as ones which have produced ascites containing tumour cells such that if small quantities of the fluid so produced are inoculated intraperitoneally into other healthy mice, peritoneal fluid containing tumour cells will accumulate.

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This paper is divided into two parts—the first consists of general observations on the later stages of development of the 7th and 8th passages of an ascites tumour obtained from a strain of subcutaneously transplanted Sarcoma 37. The second part describes experiments on the early stages of development of the same tumour in the 9th to the 12th passages. The experiments indicate the mechanism of accumulation of ascitic fluid and support the contention that ascites tumour cells multiply in intra-abdominal fatty tissues before the occurrence of an increase of cells and fluid in the peritoneal cavity.

MATERIALS AND METHODS

Sarcoma 37.—Mice bearing subcutaneous tumours of Sarcoma 37 were received from Dr. Franks of the Banting Institute, Toronto. The tumours were removed, cut finely with scissors, ground in a pestle and mortar and made into an approximately 5 per cent suspension in saline. Volumes of 0·2 ml. were injected intraperitoneally into mice. When abdominal swelling occurred, in this and subsequent passages, the peritoneal cavity was opened, fluid removed and injected intraperitoneally into further mice in volumes ranging from 0·05 to 0·5 ml. In the 7th and 8th passages, which are described in the "General Observations" section, 31 mice were inoculated with from 1·3 to 10·4 million tumours cells. The mice were killed 5 to 10 days after inoculation.

Mice.—Connaught strain white mice were used throughout. In the 1st to the 8th passages 6 to 10 week old mice were used and the sexes were segregated. From the 9th to the 12th passages only males from 6 to 8 weeks of age were used.

Cell counts.—Tumour cells were counted in a haemocytometer. Ascitic fluid was diluted 1 in 20 in saline containing methyl violet. Tumour cells were recognized by their large size and by the large, usually irregular nucleus and by the almost invariable presence of spherical shining granules in the cytoplasm.

Measurement of the volume of ascitic fluid.—Volumes of ascitic fluid greater than 2·0 ml. were measured in a 10-ml. measuring cylinder. Either a 0·2-ml. pipette graduated in 0·001 ml. or a 1·0-ml. pipette graduated in 0·01 ml. was used for smaller volumes. The point of the pipette was placed in the interstices of the intestines and run over the peritoneal surfaces so that as much fluid as possible was collected. In normal male mice aged from 6 to 10 weeks it was not possible to obtain more than 0·05 ml. of fluid.

Histological sections.—From mice described in the "General Observations" section, tissues were taken as indicated by naked eye observation at post-portem. From each mouse described in the "Experimental" sections the entire pancreatic area and associated peritoneal folds were dissected out. Both of the fat bodies attached to the testes and a piece of anterior abdominal wall, excluding skin and subcutaneous tissues were removed. The tissues were fixed in Helly's fluid and sections prepared in the usual way were stained with haematoxylin and eosin. As will be mentioned in the appropriate part of the results, serial sections were cut from many of the blocks and every tenth was stained and examined.

GENERAL OBSERVATIONS

Results

All 31 mice examined had excess fluid in the peritoneal cavity ranging from 0·25 to 8·0 ml. in volume. Counts were performed on the fluids of 14 mice. In 13 the total number of tumour cells was increased over those inoculated. The

ascitic fluid from the mouse in which there was no increase of cells was haemorrhagic, contained clumps of tumour cells and " soft tumour " (to be described below). Smears revealed a large proportion of inflammatory and other non-tumour cells, sometimes greater than that of the tumour cells.

" *Soft tumour* ".—The most remarkable and frequent observation was the presence of a whitish-yellow opaque mass in or protruding from the region lying caudal to the liver and stomach and bounded on either side by the spleen and duodenum. Gentle dissection revealed that these masses lay among the peritoneal folds that contained the pancreas. The masses were soft and friable and occurred in 19 of the 31 mice-examined. Occasionally (in 3 out of 31 mice) similar masses were seen in the substance of the bodies of fat attached to the testes ; here they were seen as yellowish opaque areas contrasting with the greyish white translucency of the normal fat. Masses of a similar appearance and consistency were frequently seen lying free in the peritoneal cavities of mice that had been inoculated with large numbers of cells or where a long time had elapsed since inoculation with smaller numbers.

Histological examination of these masses which are referred to as " soft tumour ", showed that the greater part of them was completely avascular and consisted of rounded cells with a pinkish blue opaque cytoplasm and darkly staining nuclei ; mitoses were not infrequent. These were compared with those appearing in the substance of tissues. The latter were approximately oval or polygonal in shape, with a comparatively transparent and delicately basophilic cytoplasm. The nucleus was large and vesicular. In fact the cells of " soft tumour " resembled those in ascitic fluid more closely than those apparently growing in tissues. Fig. 1 shows soft tumour from the pancreatic area lying adjacent to growth occurring in and replacing fat ; the difference between the two types of cell may be seen.

Occasionally, in sections of " soft tumour " lying free in the peritoneal cavity, there was a small vascular core of tumour tissue in the centre of the mass of rounded cells. The periphery of some of those " soft tumours " taken from the pancreatic area was bounded by peritoneum. The " soft tumours " occurring in the gonadal fat bodies were slightly different in their histological picture in that they appeared to consist of an aggregation of rounded cells within oedematous interstitial tissue.

Growth in fatty tissue.—The second observation of interest was the invariable appearance of tumour growth in histological sections of intra-abdominal fatty tissue. Sections were made from the fatty tissue of 22 mice and in every one vascular tumour could be seen. Most of the sections were taken from the region of the pancreas which, in the mouse, consists of separate lobes invested by two layers of peritoneum. In between the lobes of the pancreas the two layers of peritoneum fuse as one membrane to be separated in places by blood vessels and islands of fat invisible to the naked eye. Some sections were made from the bodies of fat attached to the gonads. At their point of attachment these bodies are of small circumference but towards their free ends they enlarge and split up into a number of processes separated by deep clefts.

Growth in the fatty tissue (Fig. 2) appeared to be diffuse, replacing fat cells, but did not seem to extend beyond the limits of the region originally occupied with fat. Cells beyond those limits were rounded and not vascularized and did not appear to take part in the tumour formation within the fat (Fig. 3). Sometimes the growth in the fat bodies appeared to spread by direct continunity along

the visceral peritoneal membranes (Fig. 4), occasionally apparently replacing vessel walls (Fig. 3). The distinction between the visceral peritoneal growth and that occurring on the parietal peritoneum (described below) should be noted.

Peripancreatic oedema.—On one occasion the pancreas was seen to be surrounded by a relatively large quantity of crystal clear gelatinous material. Histological section showed this to consist of markedly oedematous peripancreatic tissue limited peripherally by peritoneum (Fig. 5). Subsequently the pancreatic area was examined for the presence of oedema which was seen fairly commonly. Sometimes in the oedematous peripancreatic tissues numerous tumour cells were

EXPLANATION OF PLATES

FIG. 1.—34/55–5 12. Section of "soft tumour" in the pancreatic region of a mouse 5 days after inoculation with 6·4 million tumour cells. On the left tumour may be seen replacing fatty tissue. The remains of a few fat cells are shown. Towards the right the cells are rounded and not organized as vascular tumour. Cell nuclei become progressively smaller and darker and the cytoplasm more readily seem towards the right. × 85.

FIG. 2.—24/55–4 9. Section of peritoneal fat from a mouse 5 days after inoculation with 10·4 million tumour cells. Permeation between and replacement of fat cells is seen together with sheets of tumour tissue. × 85.

FIG. 3.—39/55–11 6. Section of peritoneal fat from a mouse 46 hours after inoculation with 3·1 million tumour cells, showing replacement of fatty tissue with tumour cells. There is also an intense infiltration with inflammatory cells. Rounded cells can be seen at the margin of the tissues and at the lower left hand corner is a vessel whose wall is replaced by tumour cells. × 145.

FIG. 4.—24/55–2 8. Section of fat and peritoneal membranes from a mouse 5 days after inoculation with 10·4 million tumour cells. Small islands of fat are shown replaced by tumour growth which extends along the intervening peritoneal membranes. × 85.

FIG. 5.—38/55–10 4. Section of pancreas from a mouse 6 days after inoculation with 6·6 million tumour cells. The peripancreatic tissues are markedly oedematous. No tumour cells are to be seen. The peritoneal surface is at the top. × 42.

FIG. 6.—30/55–17 7. Section of pancreas from a mouse 5 days after inoculation with 3·1 million tumour cells. Tumour and inflammatory cells are shown in the oedematous peripancreatic tissues. The tumour cells are of the rounded type and not part of organized growth. × 210.

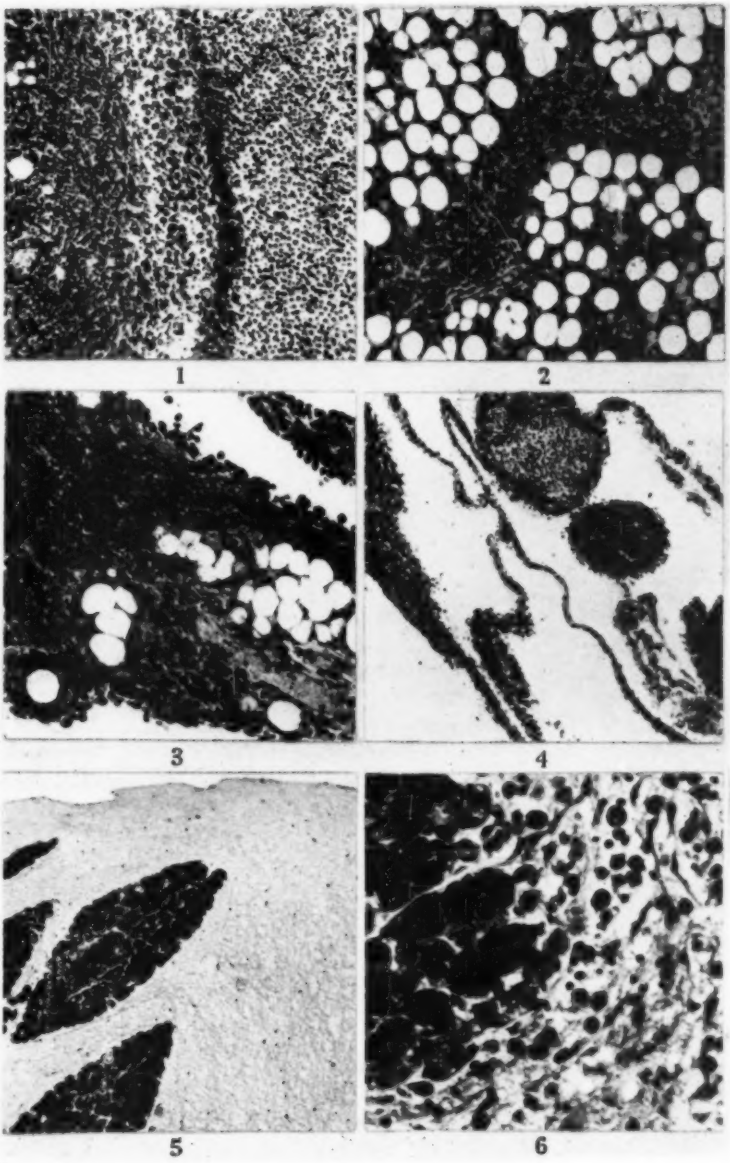
FIG. 7.—24/55–4 9. Section of parietal peritoneum from a mouse 5 days after inoculation with 10·4 million tumour cells. Heavy infiltration of the muscle with tumour and inflammatory cells is shown. Note the marked vascular dilatation. The white line running transversely across the upper part represents the original peritoneal surface on which there is a thick vascular layer of tumour and inflammatory cells. An occasional rounded cell is seen on the surface. × 85.

FIG. 9.—39/55–1 (2) 11. Section of peritoneal fat from a mouse 6 hours after inoculation with 3·1 million tumour cells. A single tumour cell is shown within the tissue applied to a fat cell wall. There is also marked vascular dilatation and infiltration with inflammatory cells. × 425.

FIG. 10.—39/55–1 (1) 10. Section of peritoneal fat from a mouse 6 hours after inoculation with 3·1 million tumour cells. Dense infiltration with inflammatory cells is shown with marked vascular dilatation. Among the inflammatory cells are larger ones which may be tumour cells. There are one or two rounded cells at the periphery of the fat. × 425.

FIG. 11.—39/55–3 (1) 1. Section of peritoneal fat from a mouse 9 hours after inoculation with 3·1 million tumour cells. Shows a dense inflammatory focus at the tip of one of the processes of a testicular fat body. To the right of the focus there is a single tumour cell lying between three fat cells. × 300.

FIG. 12.—40/55–1. Section of peritoneal fat from a mouse 18 hours after inoculation with 3·1 million cells. Shows part of a testicular fat body in which there is most of a large and part of a smaller area of interstitial oedema occupied by rounded tumour cells. Within the tissue itself there are numerous tumour cells lying between the fat cells. In the upper right portion there is a small group of tumour cells. At the top on the left dilated capillaries may be seen, one with a tumour cell applied to it. × 150.



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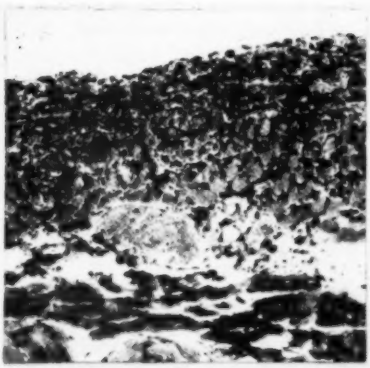

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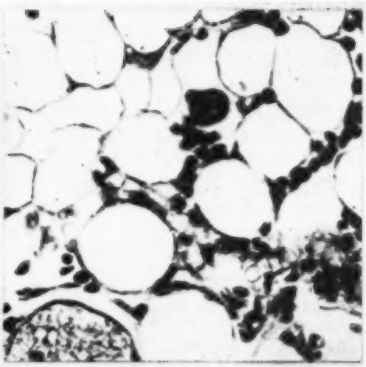

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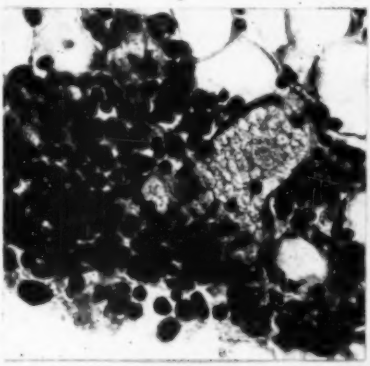

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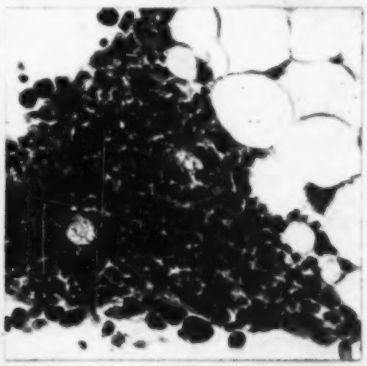

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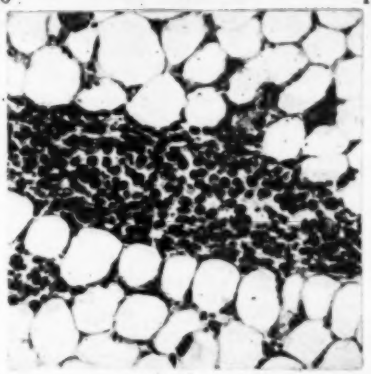

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seen (Fig. 6). The cells were of the rounded type seen in ascitic fluid and "soft tumours". No blood vessels were seen in association with them. Such collections of cells were not considered to constitute tumour growth.

Parietal peritoneal growth.—On occasions the parietal peritoneum showed a roughened pebbly appearance. Sections revealed thin layers of vascularized growth occurring on and in the parietal peritoneum (Fig. 7). Although tumour growth frequently appeared in the anterior abdominal wall at the site of inoculation it was not observed to spread in the parietal peritoneum from that site.

Fourteen sections of the spleen and 10 of the liver were taken from 14 mice with ascites during the 1st to the 12th passages none of them showed growth within or on the surface of the organs.

EXPERIMENTAL I

As a result of the general observations which were made on relatively advanced ascites tumours, experiments were planned to study the early development to see what relationship the findings had to the appearance of an increase of fluid and cells in the peritoneal cavity.

Method

Four experiments were carried out in the 9th to the 12th passages in which 70 mice were inoculated with from 3·1 to 6·6 million tumour cells. After inoculation mice were killed at intervals varying from 6 hours to 7 days. Post-mortems were performed on the 70 mice and the amount of peritoneal fluid measured. Histological sections were examined for the presence of tumour cells in both testicular and peripancreatic tissues, peripancreatic oedema and tumour growth on the parietal peritoneum. Of a possible total of 280 examinations, 14 were not carried out because specimens were either lost or inadequate.

Results

The results are shown in Tables I and II and illustrated in Fig. 8.

Growth in fatty tissue.—From the tables it may be seen that histological examination revealed tumour cells within fatty tissue in every mouse including those in which there was no increase of peritoneal fluid. Fig. 8 shows that it was the earliest phenomenon to be detected. In the early stages when tumour cells were single or in small groups, they were recognized and differentiated from normal normal cellular constituents by their very large size, clear basophilic cytoplasm and large vesicular nucleus. They were not rounded but approximately oval or polygonal in shape. Some could be seen applied to blood vessels, some to fat cells and some were in mitosis. As time went on single tumour cells first became more numerous and then appeared in groups replacing fat cells and finally as vascular sheets of growth. Often, where growth occurred in the testicular fat bodies, there were nearby regions of interstitial oedema in which rounded tumour cells were present.

Peripancreatic oedema.—Peripancreatic oedema was seen in sections from some mice killed during the earliest period and frequently in mice without excess peritoneal fluid. Fig. 8 shows that on the average it occurred after the detection of tumour cells in fat and before the appearance of ascites. With the exception of 3 mice peripancreatic oedema was always present when there was excess fluid

TABLE I.—*A Comparison of the Times of Appearance of Tumour Growth, Peripancreatic Oedema and Ascites in Mice Inoculated with Sarcoma 37 Ascites Tumour*

Time after Inoculation (hours)	Tumour cells in fat	Peri-pancreatic oedema	0·1 ml. or more of ascitic fluid	Parietal peritoneal growth
0–24	15/15	4/15	0/15	0/15
25–48	13/13	10/13	2/13	0/13
49–72	11/11	11/11	7/11	5/11
76–168	31/31	24/26	31/31	22/29
Total	70/70	49/65	40/70	27/68

Numerators = number of mice affected.
Denominators = number of mice observed.

TABLE II.—*The Relation between Ascites Production and the Appearance of Tumour Growth and Peripancreatic Oedema in Mice Inoculated with Sarcoma 37 Ascites Tumour*

Relation to appearance of 0·1 ml. or more of ascitic fluid	Tumour cells in fat		Peri-pancreatic oedema	Parietal peritoneal growth
	Pancreatic area	Testicular bodies		
Before	29/29	29/30	17/30	1/30
After	35/35	39/39	32/35	26/38
Total	64/64	68/69	49/65	27/68

Numerators = Number of mice affected.
Denominators = Number of mice observed.

in the peritoneal cavity. The 3 exceptions occurred in mice with small amounts of ascitic fluid of 0·25 ml. or less.

In oedematous peripancreatic tissue, rounded cells were scanty or absent in mice killed in the early stages. As time went on they became more and more numerous. At no time were collections of these rounded cells seen to be vascularized.

Parietal peritoneal growth.—Infiltrating vascularized tumour growth on the parietal peritoneum occurred only after 48 hours (Table I) and, with one exception, was always accompanied by excess peritoneal fluid (Table II). Thus, in general, growth on the parietal peritoneum occurred after the production of ascitic fluid (Fig. 8).

Soft tumour.—In this series of animals "soft tumour" was never seen before the 4th day nor did it appear in the testicular fat bodies. On the 4th day 3 out of 12 animals were affected: one had "soft tumour" in the pan-

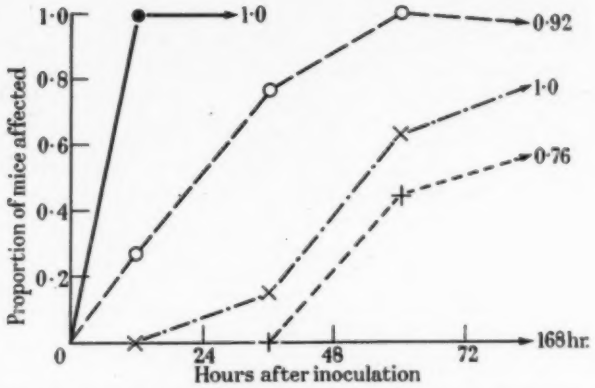

FIG. 8.—Graphic representation of data from Table I. The sequence of changes occurring in the development of Sarcoma 37 ascites tumour.

Abscissa : Time in hours after inoculation.
Ordinate : Proportion of mice showing :
(*a*) Tumour cells in fatty tissue.
(*b*) Peripancreatic oedema.
(*c*) 0·1 ml. or more of peritoneal fluid.
(*d*) Parietal peritoneal growth.

creatic region only and one had it lying free. The other mouse had it in both sites. After the 4th day, 17 out of 19 mice were affected. Ten had " soft tumour " in the pancreatic region only, 2 had it lying free and 5 had it in both places. The ascitic fluid of those mice with " soft tumour " was usually haemorrhagic. In 9 of the 10 counts performed the total number of tumour cells in mice with " soft tumour " was markedly lower than that in fluids from mice killed earlier and sometimes was less than the number inoculated.

EXPERIMENTAL II

The mice in one of the experiments described above were examined in greater detail and the salient features have already been reported in brief (Warner, 1955).

Method

Twenty-one mice were inoculated with 3·1 million sarcoma cells and groups of them were killed during varying periods after inoculation. In addition to the investigations described in " Experimental I " cell counts were performed on each peritoneal fluid.

Results

The results are shown in Table III. During the first 22 hours there is no significant increase in the total number of tumour cells over those inoculated and no accumulation of peritoneal fluid. In fact, since it was possible to collect barely enough fluid to provide for that (0·02 ml.) required for the tumour cell count, it is possible that there was a diminution in the total number of tumour cells over those inoculated. However in each mouse killed during this period tumour cells were detected in sections of intra-abdominal fatty tissue.

Tumour cells were scanty in sections from mice killed between 6 and 10 hours and sometimes could only be seen (Fig. 9) after prolonged examination of 20 to 30 sections of each tissue. The earlier sections showed inflammatory cells in dense foci in which vascular dilatation was marked (Fig. 10). It was noticed that when they were scanty tumour cells could be seen clearly only near or at the edge of the foci (Fig. 11). Within the inflammatory foci, cells, which were thought to be tumour cells, were seen but, owing to the dense infiltration of inflammatory cells, they were not sufficiently distinct for their nature to be determined for sure (Fig. 10). In mice killed between 18 and 22 hours tumour cells were more numerous sometimes occurring in small groups.

TABLE III.—*The Relation between Increase of Cells and Fluid and the Appearance of Tumour Growth and Peripancreatic Oedema in Mice Inoculated with Sarcoma 37 Ascites Tumour*

Time after inoculation (hours)	Mouse number	Tumour cells (millions per ml.)	Volume ascitic fluid (ml.)	Total tumour cells (millions)	Average tumour cells (millions)	Tumour cells in fat	Peri-pancreatic oedema	Parietal peritoneal growth
6–10	1	33·4		<3·3		+	0	0
	2	28·1		<2·8		+	0	0
	3	24·2	<0·1	<2·4	<2·9	+	0	0
	4	32·3		<3·2		+	0	0
	5	28·5		<2·9		+	0	0
18–22	6	24·4		<2·4		+	+	0
	7	24·2	<0·1	<2·4	<2·5	+	+	0
	8	30·3		<3·0		+	0	0
	9	22·3		<2·2		+	0	0
30–48	10	48·4		<4·8		+	+	0
	11	36·2	<0·1	<3·6		+	0	0
	12	53·0		<5·3		+	+	0
	13	46·5	0·25	11·6	<6·8	+	0	0
	14	69·4	0·1	6·9		+	+	0
	15	84·8	<0·1	<8·5		+	+	0
72	16	118·7	0·1	11·9		+	+	0
	17	47·8	0·2	9·6	11·2	+	+	+
	18	40·4	0·3	12·1		+	+	+
96	19	28·9	0·3	8·7		+	+	+
	20	33·0	0·6	19·8	10·0	+	+	+
	21*	5·4	0·3	1·6		+	+	+

\+ = Present.
0 = Absent.
* = This mouse has soft tumour lying free in the peritoneal cavity.
All mice were inoculated intraperitoneally with 3·1 million tumour cells.

Between 30 and 48 hours there was probably, on the average, an increase in the total number of tumour cells over those inoculated. In sections of fatty tissue from all mice killed during this period groups of sarcoma cells were present and small vascular sheets of them replaced fatty tissue.

At 72 and 96 hours there was a definite increase of cells and peritoneal fluid. However in mouse 21 the total number of cells was less than the number inoculated ; this mouse had soft tumour lying free in the peritoneal cavity. Sections from all mice killed during this period showed vascular sheets of sarcoma replacing relatively large areas of fatty tissue, similar to those shown in Fig. 2.

DISCUSSION

The most important part of the present investigation is that tumour cells were found in intraperitoneal fatty tissue before an appreciable increase of cells or fluid occurred in the peritoneal cavity of mice inoculated with Sarcoma 37 ascites tumour. Therefore it is important to consider two points in the techniques used in arriving at this conclusion.

As far as the measurement of small amounts of fluid is concerned it was only possible to obtain quantities less than 0·1 ml. from normal male mice, it was considered sufficiently accurate for the purpose to regard volumes of 0·1 ml. or more as a criterion of increased production of fluid. Using this criterion it was found that on the average, fluid increase was correlated with the increase in total number of tumour cells (Table III). This finding supported our view as it is in keeping with that of other workers (Klein and Revesz, 1953 ; Patt and Blackford, 1954).

The other important point to be considered is the recognition of single tumour cells. This is notably difficult, but they could be seen clearly in the sparsely nucleated fatty tissue and were felt to be sufficiently distinctive to diagnose confidently as tumour cells. In any event there was no difficulty in recognizing foci of tumour cells at a later stage. It was felt that the presence of foci was compatible with the finding of single cells at an earlier stage.

As a result of these considerations and the fact that all mice with ascites showed clearly recognizable tumour in fatty tissue, it was concluded that the growth of this ascites tumour took place in tissues before an increase of tumour cells and fluid occurred in the peritoneal cavity. In addition since growth was not observed on or in the liver and spleen and only at a later stage on the parietal peritoneum, it was concluded that early invasion of fatty tissue was an invariable characteristic of this tumour. Furthermore our observations are compatible with the hypothesis that tumour cells are liberated from growth foci in fatty tissue and pass into the peritoneal cavity. Hence, growth foci in tissues could be the sole source of the increasing number of tumour cells in suspension in peritoneal fluid.

The results show that tumour cell infiltration of fatty tissue was followed by peripancreatic oedema which preceded the appearance of ascitic fluid. It is reasonable to conclude that tumour growth in the region of the pancreas causes oedema by interfering with the local vascular supply. This is supported by the observation that tumour cells replace vessel walls. The oedema fluid thus produced may be assumed to pass into the peritoneal cavity thereby producing ascites. The occurrence of oedema in the testicular fat bodies under similar circumstances suggests that the same sequence of events may occur there. It is also possible that the inflammatory reaction occurring soon after the inoculation of tumour cells, through markedly dilated blood vessels (Fig. 9, 10 and 11), contributes to the ascites. At a later stage when the peritoneum is widely involved with vascular tumour tissue (Fig. 7), fluid may pass from its altered surface into the cavity. Although it is not contended that asciitic fluid collects by one of these mechanisms only it seems most likely that in the early stages the main contribution is from the peripancreatic area where interference with the main radicles of the portal vascular system is particularly liable to produce ascites.

No progressively enlarging solid tumour growth comparable to that occurring in subcutaneously transplanted Sarcoma 37 was observed within the peritoneal

cavities of mice used in this investigation. Where tumour growth occurred in fat it replaced normal tissue but did not extend as vascular tumour beyond its confines. It was concluded that "soft tumours" were merely aggregates of, free tumour cells liberated from foci of growth in fatty tissue and, in many instances, trapped by peritoneal folds. Aggregation of cells would diminish the number in suspension and thus explain the low tumour cell counts obtained when "soft tumour" was present. "Soft tumours" lying free in the peritoneal cavity could have been aggregates of cells formerly in suspension, but some of them may have been detached from the pancreatic region which could explain the presence of vascular cores in them and the concomitant haemorrhage. Observation on a phenomenon similar to "soft tumour" occurring in the Ehrlich ascites tumour are described by Bailliff (1954) who uses the term "semi-solid" tumour.

It follows from our conclusions that it is not necessary to assume multiplication of Sarcoma 37 cells while they are in suspension in peritoneal fluid. This conflicts with the views of Goldie and Felix (1951) and Klein (1951); it may be that these findings apply only to Sarcoma 37 or the strain of mice used; or, it may be claimed that in twelve passages the tumour had not become adapted to the intraperitoneal environment sufficiently well for the cells to multiply in peritoneal fluid; or the findings could be attributed to the number of tumour cells inoculated being too low to produce typical ascites tumours. Against these objections it may be said that Sarcoma 37 is not a discriminating tumour and it grows readily in many strains of white mice. Also the number of passages and tumour cells inoculated are both in excess of those used by Goldie and Felix (1951) to obtain the ascites form of the same tumour.

On *a priori* grounds one would expect what is in fact a graft of tumour cells to behave in the peritoneal cavity in approximately the same way as in subcutaneous tissues. Warner, Gostling and Thackray (1950) have provided evidence that the majority of cells in a subcutaneous graft of Sarcoma 37 becomes necrotic; a few cells survive and pass from the graft into the host tissues where they multiply in the neighbourhood of blood vessels from which, presumably, they obtain the necessary nutriment. The findings described in this paper suggest that a similar sequence of events may occur in the ascites form of the same tumour. Thus, the formation of new vessels is unnecessary for the growth of the tumour because the cells find their way to the neighbourhood of already existing vessels. Hence the claim of Goldie and Felix (1951) that they could detect an increase of Sarcoma 37 ascites tumour cells in peritoneal fluid before vascularization could have taken place is not sufficient to support their view that multiplication takes place while the cells are in suspension.

In contrast to the findings described in this paper, Lasnitzki (1953) using Sarcoma 37 ascites tumour concluded that round free cells grew more rapidly than spindle cells that were morphologically similar to those occurring in tissues. She found that, when free ascites tumour cells were embedded in a plasma clot, at first they remained round but after incubation for 24 hours they became spindle shaped. Subcutaneous grafts in mice of these spindle cells produced fewer tumours and grew more slowly than grafts of round cells. However, the spindle cells had been incubated for 24 hours whereas the round cells had not and this circumstance alone may have accounted for the slower growth of spindle cell grafts. In further support of her contention, Lasnitzki (1953) states that, in plasma clot, mitoses were seen in round cells only. However, the presence of

mitotic figures in cells does not necessarily mean that they are actively dividing. A mitotic figure may only indicate the state of a cell at the moment of its removal from an environment suitable for growth. While the cell is in suspension or otherwise removed from a suitable environment, it may remain in a state of suspended animation or dormancy in which the mitotic figure remains stationary. Furthermore it has never been shown that " free " or suspended cells will increase in number in stationary tissue culture and will only do so under special conditions in moving cultures. It is a common observation, during the initiation of cultures on glass of such tissue as HeLa cells, monkey kidney tissue and human amnion, that the inoculated cells are rounded. The rounded cells become applied to the glass surface and spread out becoming spindle shaped or polymorphous. In such cultures, if the cells remain round they do not thrive, whereas spindle cells increase in number. For growth in tissue culture, cells apparently need to become attached to some support and require adequate nutriment. That similar conditions obtain *in vivo* is suggested by Lasnitzki's (1953) observations on histological preparations of subcutaneous grafts. The central portion of her grafts, which eventually became vascular tumour tissue, consisted of spindle cells. At first no growth was detected in these cells which could, by analogy with tissue culture, be regarded as being attached to a supporting structure. At a later stage, when blood vessels appeared (presumably carrying adequate nutriment), growth of the spindle cells occurred. It is easy to see that a similar explanation can be applied to the development of the ascites form of the same tumour.

At present the only crucial finding supporting a hypothesis that multiplication of tumour cells takes place in suspension would be that in which there is an increase in the number of cells combined with the absence of growth in tissues. The ability to detect tissue invasion depends on the intensity of the search. Where tumour foci in tissues are few and small in size or possibly, as in this work, obscured by inflammatory cells, the probability of detecting one may be small. In fact it would be necessary to examine serial sections of each piece of tissue before one could say with confidence that tumour growth was absent. Dealing with this matter in connection with Sarcoma 37 ascites tumour, Klein (1951) states that he detected an increase of cells in the peritoneal fluid before he could find tumour cell infiltration of tissues. He does not indicate how many sections were taken from each tissue, nor how many tissues from each mouse, nor is there clear correlation between tumour cell counts of individual mice and histological findings. Thus it is impossible to judge the intensity of his search for foci of tumour growth. Therefore there remains the possibility that an insufficient number of sections were examined to reveal early tumour growth in tissues; consequently his results are not necessarily in conflict with those described here.

If the arguments put forward are correct then it is possible that the findings described here may have a more general application to other ascites tumours. The finding of growth of the Ehrlich (Bailliff, 1954; Klein, 1951) and the Krebs (Krebs, Thordarson and Harbo, 1942) ascites tumours in fat and the description of something akin to " soft tumour " in the Ehrlich ascites tumour (Bailliff, 1954) indicate similarity between these and the Sarcoma 37 ascites tumour. Even if tissue growth is demonstrated before the occurrence of an increase in the number of cells in the peritoneal fluid in ascites tumours in general, there would still remain the question of whether any cell multiplication at all takes place in peritoneal fluid. Such findings as an exponential increase in the number of cells in suspension

or changes in mitotic rate can be explained by assuming that cells are liberated from growing foci in tissues. In fact all the described phenonomena exhibited by ascites tumours can be satisfied by the hypothesis that growth occurs only in tissues, and the production of ascitic fluid can be more satisfactorily explained by this hypothesis than by that of growth in suspension. Thus, to conclude, it is unnecessary to assume that ascites tumour cells multiply in suspension in peritoneal fluid.

SUMMARY

This paper describes observations on 101 Connaught strain white mice after inoculation with Sarcoma 37 ascites tumour in the 7th to the 12th passages.

Post-mortem examinations of 31 mice with well-developed ascites tumours showed no tumours similar to the subcutaneous form but soft avascular masses of aggregated tumour cells frequently occurred. Histological sections of intra-abdominal fatty tissue from 22 mice all showed diffuse invasion with tumour tissue.

Seventy mice were killed at intervals from 6 hours to 7 days after inoculation with 3·1 to 6·6 million tumour cells. Sections of fatty tissue from all mice showed the presence of tumour cells; 30 of the mice had not developed appreciable ascitic fluid. Tumour cell counts were performed in 9 mice within 22 hours of inoculation and there was no increase in number over those inoculated. Seventeen out of 30 mice without appreciable ascites and 32 out of 35 with ascitic fluid showed peripancreatic oedema. The three mice in the latter group had only 0·25 ml. or less of ascitic fluid. Parietal peritoneal growth occurred relatively late in the development of ascites tumours and was observed only once in 30 mice without ascitic fluid and in 26 out of 38 mice with ascitic fluid. Aggregated tumour cells were present in some mice from the 4th day onwards.

From the results it was concluded that growth of Sarcoma 37 ascites tumour occurred in fatty tissues before an appreciable increase of cells or fluid in the peritoneal cavity. Growth in peripancreatic fatty tissue interfered with the portal circulation causing oedema which contributed fluid to the ascites. Foci of growth liberated cells into the peritoneal fluid. Liberated cells often become aggregated to form tumour-like masses but no true tumour formation took place. In view of these results it is unnecessary to assume that ascites tumour cells multiply within peritoneal fluid.

The authors are indebted to Miss L. Nason for the preparation of the photomicrographs and to Mr. G. A. Gormly for the preparation of Fig. 8. This work was supported by a grant from the National Cancer Institute of Canada.

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ADRENAL DEGENERATION AND TUMOUR FORMATION IN THE GOLDEN HAMSTER FOLLOWING TREATMENT WITH STILBOESTROL AND METHYLCHOLANTHRENE

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Received for publication January 29, 1957

THE histological pattern of the adrenal cortex of the golden hamster is similar in most respects to that of other Eutherian mammals (Bourne, 1949) and has been described by a number of workers (Peczenik, 1944; Koneff, Simpson and Evans, 1946; Keyes, 1949; and others). In contrast to most other species the cortical cells contain little or no sudanophil or osmophil lipid or cholesterol (Wexler, 1951; Agate, 1952; Knigge, 1954). Three zones, glomerulosa, fasciculata and reticularis, are present, but the distinction between fasciculata and reticularis is not always well defined. Holmes (1955) has also described an X zone in females, and in some animals of either sex a zona intermedia between the glomerulosa and fasciculata can be seen (Chesterman, unpublished). Peczenik (1944) described a zone of vacuolated cells in the zona fasciculata but these vacuoles did not contain stainable lipid. The vacuoles increased in size with sexual maturity. In the breeding season in males the vacuolated zone was seen in the outer layers of the fasciculata but in females most of the cells of the fasciculata were vacuolated. In non-breeding animals the vacuoles were scanty or absent.

In a few senile animals the reticularis and inner layers of the fasciculata were more vacuolated than the outer. Peczenik also described the changes which follow castration and the administration of oestrogens and other hormones for relatively short periods (3–4 weeks). The purpose of the present paper is to describe some adrenal changes which follow treatment with stilboestrol for longer periods (1–15 months). The experiment was planned to study the effects of stilboestrol and methylcholanthrene on the kidneys and prostate. The adrenal changes were an incidental finding.

MATERIAL AND METHODS

Adrenal glands from 94 hamsters were available for study, from five groups:

1. Thirty-six untreated animals.
2. Eighteen animals treated with stilboestrol.
3. Twenty-one animals treated with stilboesterol and in addition, implanted with methylcholanthrene (approximately 1·0 mg. in Lubafax jelly) in the dorsal prostate.
4. Four animals treated with methylcholanthrene alone.
5. Fifteen animals treated with other hormones, with or without methylcholanthrene.

The stilboestrol was administered as a 15 mg. pellet (Implantin, Burroughs Wellcome) which contained 1 per cent magnesium stearate, the remainder being pure stilboestrol. Implants were repeated as necessary—generally at 3-monthly intervals but sometimes longer.

The age range and length of treatment is given in Table I.

TABLE I.—*Age Range, Sex and Treatment: 94 Experimental Animals*

Treatment	Sex	Number of Animals	Age range (months)	Length of treatment
None	M.	27	6–24	—
	F.	9	4–18	—
Stilboestrol	M.	10	4–14	1–11
	F.	8	3–18	2–12
Stilboestrol and methylcholanthrene	M.	21	3–19	3–15
Methylcholanthrene	,,	4	4–11	6–24
Miscellaneous	,,	15	10–12	7–17

The glands were fixed in neutral formalin, embedded in paraffin and sections were stained with Ehrlich's haematoxylin and eosin and Bennhold's (1922) Congo red method for amyloid.

RESULTS

There was no appreciable sex difference in the adrenal cortex, nor was vacuolation a marked feature in either males of females (Fig. 1) even in the breeding season. Stilboestrol, however, produced a characteristic change which generally began in the middle zone of the zona fasciculata. This change was similar to that described by Peczenik (1944) and Koneff, Simpson and Evans (1946), although ultimately more severe. After one month, isolated pale staining cells with finely vacuolated cytoplasm and rather darkly staining nuclei could be seen in the zona fasciculata. After 3 months these cells were much more frequent, and large cells with abundant cytoplasm and large nuclei lay between the cords of the zona fasciculata. In addition a zone in the middle of the fasciculate layer showed nuclear irregularity with variation in shape, size and intensity of staining. The cytoplasm was often eosinophilic. By 6 months the changes were well developed. The normal trabecular arrangement of the adrenal cells was lost, the cords being replaced by irregular masses of large cells with eosinophil, sometimes coarsely granular, cytoplasm and large irregular nuclei. There were many bizarre and irregular cells. In some the cytoplasm was foamy and finely vacuolated; in others the vacuoles were large (Fig. 2). The nuclei were sometimes pyknotic and fragmented. This change at first involved only the middle zone of the fasciculata but later affected most of the gland, although as a rule the zona glomerulosa remained intact. The distinction between the zona fasciculata and reticularis was lost.

After about 6 months' treatment degenerative changes appeared in these presumably hyperplastic cells. The cells underwent an eosinophilic coagulative necrosis which at first affected only small groups in the middle of the zona fasciculata (Fig. 3 and 4) but later involved almost the whole zone and ultimately the whole gland except for the zona glomerulosa, and sometimes a few cells of the outer zona fasciculata (Fig. 5 and 6). Haemorrhage with rupture of sinusoid walls occurred in these necrotic areas (Fig. 7), and there seems little doubt that

this had followed the necrosis. The latter change in some, but not all, cases occurred about 10–14 days after re-implantation with stilboestrol and generally appeared after 7–12 months' total treatment.

A similar type of degeneration involving the reticularis and inner part of the fasciculata (Fig. 8 and 9) was seen in 11 animals. It occurred more frequently in older animals and was often associated with chronic sepsis. The surviving fasciculate cells in these animals were generally normal although the cells next to the degenerate areas often showed nuclear pyknosis. It was not seen in any animals treated with stilboestrol alone (Table II). Some of the hyaline eosinophil

TABLE II.—*Age, Treatment and Cause of Death in Animals with Degeneration Mainly in the Zona Reticularis*

Ref. No.	Treatment	Age in months	Cause of death
193	None	24	Killed.
58	None	28	Died. Pyelonephritis, pelvic abscess, urinary calculus.
86	Methylcholanthrene	28	Killed. Found in dying condition, generalised oedema.
67	Methylcholanthrene and stilboestrol	14	Died. Intestinal obstruction.
40	Ditto	15	Killed. Chronic abscess in pelvis.
71	,,	17	Killed.
66	,,	18	Died. Renal failure; prostatic abscess.
49	,,	22	Killed. Chronic ulcer of flank.
41	,,	30	Killed. In dying condition, generalised oedema.
13	Miscellaneous	21	Killed. Chronic inflammation of salivary gland and tongue.
6	,,	33	Died. No obvious cause.

material in the degenerate areas lay in the walls of the sinusoids and gave a positive reaction for amyloid (Bennhold's Congo red method, 1922). [Amyloid deposition commonly follows experimental infections in hamsters (Chute, Fenton and Sommers, 1954; and others).] Although small deposits of similar material were also seen in some of the stilboestrol-treated animals, the large degenerate areas in these glands did not as a rule take the stain.

Adrenal changes were found in all stilboestrol-treated animals, of either sex treated for more than 1 month except for 4 of 6 males segregated and treated when 3 months old, for periods of 5, 6, 10 and 12 months. The 2 other animals in this group, treated for 8 and 15 months showed slight changes only.

Tumour formation in the Adrenals

In some untreated animals there were small subcapsular areas of nodular hyperplasia apparently arising in the zona glomerulosa, although some of the cells resembled fasciculate cells. In one case there was a definite fibrous capsule around the nodule (Fig. 10). These changes were seen only in old animals (4 males, aged 18, 24, 24, and 28 months; 1 female, aged 18 months). Woolley (1953) has previously reported small adrenal tumours in untreated or gonadectomised old hamsters.

Stilboestrol-treated animals also showed localised but ill-defined areas of irregular cellular hyperplasia but these nodules arose in the zona fasciculata. In 2 male animals, however, in addition to the diffuse hyperplasia of the fasciculate cells there were also localised nodules of similar, although rather more irregular, cells (Fig. 11 and 12) which formed definite cortical tumours.

In another male animal, aged 22 months at death (treated with stilboestrol, 2 implants in 12 months, and an initial application of 1 mg. methylcholanthrene in jelly to the dorsal prostate) a large tumour, measuring 1·1 cm., in its greatest diameter, was found in the left adrenal (Fig. 13). There was a narrow rim of zona glomerulosa at one margin but the gland was largely replaced by a tumour made up of solid masses and cords of eosinophil cells in some areas resembling those of the normal adrenal, but in others the cells were more anaplastic. In parts the cells were foamy, and elsewhere small groups were similar to those seen in the inner zones of the adrenal in oestrogen-treated animals. There were areas of degeneration and haemorrhage in the tumour. The other adrenal showed the hyperplastic changes seen in other stilboestrol-treated animals but although some clumps of cells showed marked irregularity, there were no definite tumours. Many macrophages filled with iron pigment were present in this gland. Another animal, a male 33 months old at death, treated with methylcholanthrene, 1 mg. in jelly to the dorsal prostate, had a similar but smaller tumour, measuring 0·25

EXPLANATION OF PLATES

FIG. 1.—Normal adrenal, male hamster aged 19 months. × 90.

FIG. 2.—Adrenal of stilboestrol-treated male hamster showing a moderate cellular irregularity and vacuolation of the cytoplasm of some cells. (Aged 11 months; treated 7 months.) × 90.

FIG. 3 and 4.—Adrenal of stilboestrol-treated male hamster showing early degenerative changes in the zona fasciculata. (Aged 16 months; treated 6 months.) Fig. 3 × 27; Fig. 4 × 90.

FIG. 5.—Adrenal of stilboestrol-treated female hamster showing extensive degeneration (pale), of the inner zones of the gland. The medulla is unaffected. (Aged 13 months; treated 9 months.) × 27.

FIG. 6.—As Fig. 5 showing the surviving irregular adrenal cells mainly in the outer part of the zona fasciculata. (Female, aged 17 months; treated 4 months.) × 90.

FIG. 7.—Adrenal of stilboestrol-treated male showing extensive degeneration and haemorrhage (dark). (Aged 11 months; treated 7 months.) × 25.

FIG. 8 and 9.—Showing degeneration mainly in zona reticularis. The remaining zona fasciculata cells are normal. (Male, aged 22 months; treated with stilboestrol and methylcholanthrene, 3 months.) Fig. 8 × 25; Fig. 9 × 80.

FIG. 10.—Small partly encapsulated adrenal nodule possibly arising from the zona glomerulosa in an untreated male hamster 24 months old. × 80.

FIG. 11.—Adrenal of stilboestrol-treated male hamster showing two cortical nodules probably arising in the zona fasciculata. A zone of hyperplastic (dark) fasciculate cells can also be seen. (Aged 15 months; treated 11 months.) × 25.

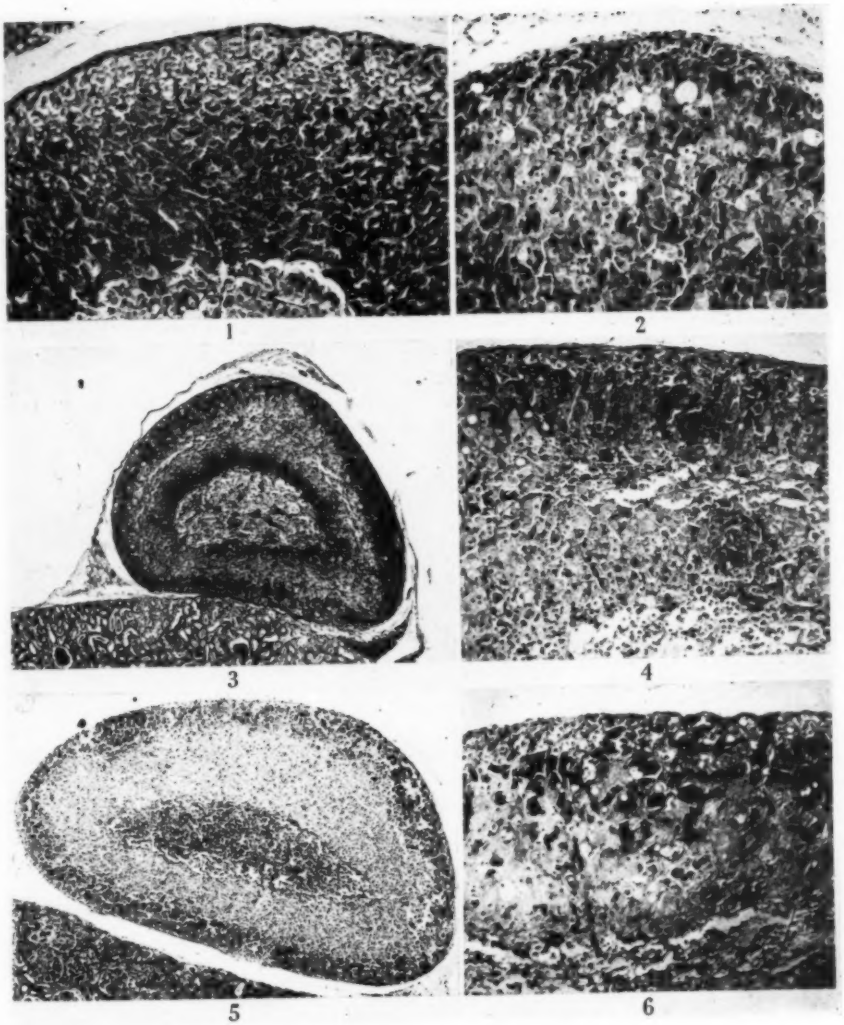
FIG. 12.—The smaller nodule seen in Fig. 11; hyperplastic fasciculate cells resembling those of the nodule can be seen at the margin. The zona glomerulosa is normal. × 80.

FIG. 13.—A large adrenal tumour in a male hamster, aged 22 months, treated with stilboestrol and methylcholanthrene for 12 months. × 6.

FIG. 14.—A smaller adrenal tumour in a male hamster aged 33 months treated with a single implant of methylcholanthrene alone, 22 months before death. × 6.

FIG. 15.—Showing the cellular structure of the tumour shown in Fig. 14. One tumour cell is in mitosis. × 550.

All sections stained by Ehrlich's haematoxylin and eosin.



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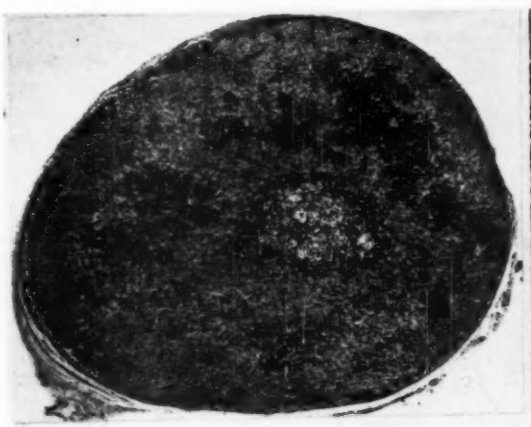

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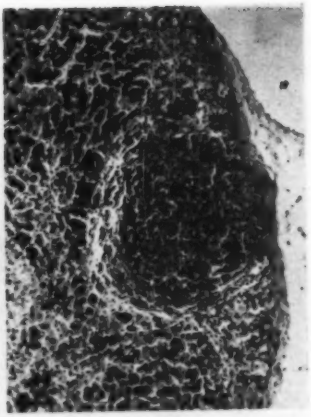

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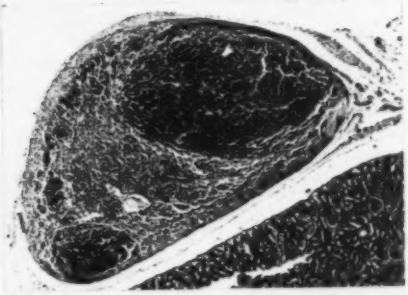

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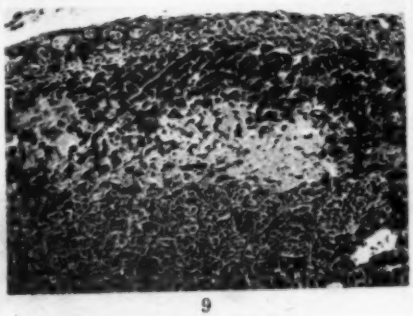

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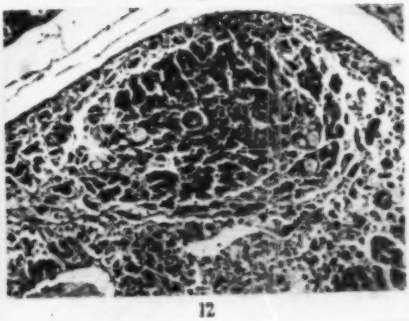

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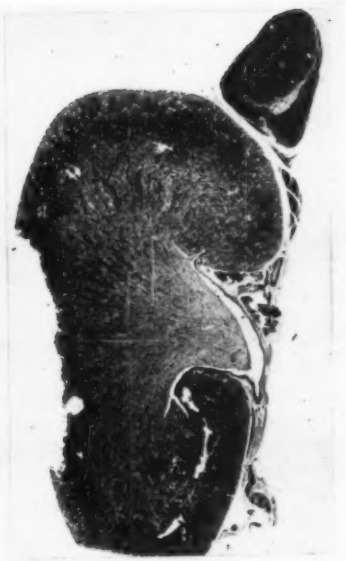

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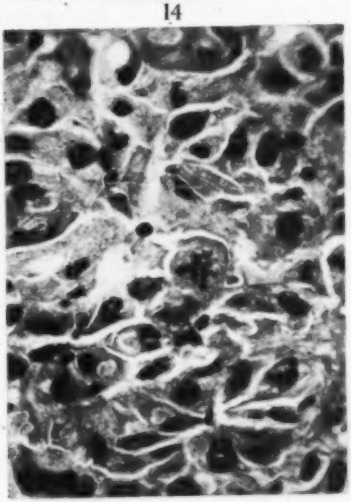

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mm. in its greatest diameter, in one adrenal (Fig. 14 and 15). The remainder of the gland was compressed at the margin of the tumour but was otherwise normal. The animal died 22 months after the application of the methylcholanthrene. No attempt was made to transplant these tumours. The changes which occurred in other organs—particularly the kidneys, prostate and pituitary—are to be described in detail later.

DISCUSSION

The changes in the adrenal which follow treatment with stilboestrol seem to begin in the middle layers of the zona fasciculata. These cells become swollen and hyperplastic, and eventually the whole of the fasciculata and reticularis is involved. Although early changes can be seen after 1 month, the process does not become marked until 3 months or more ; after 6 months, areas of degeneration, followed by haemorrhage, develop and may destroy the whole gland except for the zona glomerulosa. In retrospect it seems likely that many oestrogen-treated animals had died from acute adrenal insufficiency.

Because of the delay before marked changes appear in the adrenal, it seems likely that stilboestrol does not produce this effect by its direct action on the adrenal cells, although it is known to have an A.C.T.H.-like effect in some animals (Pincus, 1955). It is also known (Koneff, Simpson and Evans, 1946) that oestrogen treatment of hamsters leads to hyperplasia and later tumour formation in the pars intermedia of the pituitary, first becoming marked after 3½ months, and it is therefore possible that a pituitary factor may be involved. It may be that the hyperplastic changes are due to an A.C.T.H.-like effect of stilboestrol and that the degenerative changes we have observed may be due to pituitary hypofunction as a result of the tumour-like proliferation of the pars intermedia. Hypophysectomy in most species leads to atrophy of the inner zones of the adrenal, particularly involving the fasciculata, a further point in favour of this suggestion.

Selye (1947), however, illustrates a similar type of adrenal degeneration in rats sensitised by unilateral nephrectomy, castration and a high salt diet and subsequently treated with large doses of lyophilised anterior pituitary tissue. He suggests that the necrosis is produced by overstimulation. Russell and his co-workers (1941) in studies on the toxicity of stilboestrol, reported small areas of adrenal cortical necrosis in a few rats treated for up to 7 weeks, which they regarded as a toxic change.

Very similar degenerative and haemorrhagic lesions have been described in the adrenal glands of women dying during pregnancy or the puerperium (Crawford, 1951). In these cases two types of lesion were recognised—local or more extensive haemorrhages, often related to masses of " enormously swollen vesicular cortical cells ", and focal or diffuse areas of necrosis, sometimes with no evidence of previous haemorrhage. In some cases there was a fibrinous thickening of the walls of the adrenal sinusoids. As in the hamsters, the lesions appeared to involve the zona fasciculata particularly. Many of these patients were suffering from eclampsia or severe toxaemia of pregnancy.

The apparent localisation of the initial response to the zona fasciculata may be of some interest, particularly if work on the site of production of individual hormones in the adrenal can be extended. Farrel, Banks and Koletsky (1956) and Ayres *et al.* (1956) have suggested that cortisol is mainly produced in the zona fasciculata, and aldosterone in the zona glomerulosa but in our experiments

no attempt was made to estimate hormone excretion nor to estimate adrenal weights. The resemblance of some of the adrenal changes to those seen in Conn's syndrome has already been reported (Franks and Chesterman, 1956).

Furth *et al.* (1956) have reported adrenal and renal changes resembling those we have seen, in hyperoestrinised rats bearing dependent mammatrophic pituitary tumours and less marked changes in other untreated rats bearing autonomous transplanted tumours of this type. Greene (1939) has also described cortical adrenal changes in rabbits with a high familial incidence of mammary tumours. These tumours were associated with changes in the pituitary and uterus which resembled those produced by oestrogen treatment.

The tumour nodules in the adrenals are of interest because this is an unusual result of oestrogen treatment in laboratory animals (Burrows and Horning, 1952). Horning and Whittick (1954), however, have described nodular hyperplastic foci in three oestrogen-treated hamsters and mention that Gardner (1947) reported cortical adenomata in oestrogen-treated mice which also developed pituitary tumours after prolonged treatement. Dunning, Curtis and Segaloff (1953) have also reported adrenal tumours in certain inbred rat strains following treatment with oestrone or diethylstilboestrol. In our series, the 2 old animals with large adrenal tumours had been treated with methylcholanthrene. The structure of these tumours resembled that of the small cortical nodules in old untreated control animals and it is possible that these may have arisen from the cells of the zona glomerulosa. The nodules in animals treated with stilboestrol alone almost certainly developed from the cells of the inner zones of the adrenal.

Another point of note is the absence of oestrogen-induced changes in males treated when young.

SUMMARY

Stilboestrol treatment of golden hamsters is followed by the formation of large irregular, presumably hyperplastic, cells in the zona fasciculata of the adrenal. The first changes appear in the middle of this zone but later involve the whole of the fasciculata and reticularis. After about 6 months areas of degeneration and haemorrhage may develop in the zona fasciculata, leading in some cases to almost complete destruction of the cortex. This change resembles that sometimes seen in women dying in pregnancy or the early puerperium. Another type of degeneration, involving mainly the zona reticularis, was also seen; this occurred mainly in old animals and was often associated with chronic sepsis and amyloidosis. The stilboestrol-induced changes were seen in breeding animals of either sex but they were very slight or absent in segregated males treated when young (3 months).

In 2 stilboestrol-treated males there were localised tumour nodules arising from the inner zones of the cortex and in two other males, one treated with methylcholanthrene alone and the other with methylcholanthrene and stilboestrol, there were large cortical adrenal tumours. These large tumours resemble small nodules sometimes seen in old untreated animals and may be derived from zona glomerulosa cells.

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ADDENDUM

Since this paper was written our attention has been drawn to an article by M. W. Meyers and H. A. Charipper (*Anat. Rec.*, 1956, 124, 1) describing the changes in the adrenal of the aging hamster. Some of these changes resemble those produced by stilboestrol in young animals.

PSUEDO-MEMBRANES ENCLOSING NUCLEI OF DIVIDING ASCITES TUMOUR CELLS

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Received for publication January 23, 1957

THE cytological abnormalities of neoplastic cells have been studied by many workers because of their possible significance in carcinogenesis, malignancy and diagnosis. Abnormal mitotic figures, including multipolar spindles (Kemp, 1930; von Moellendorff, 1940) and absence of a spindle (Ludford, 1930, 1942), have been reported in tumour cells. They have also been seen in pre-cancerous cells (Block, 1932; Mendelsohn, 1935). Disturbances of chromosome segregation have been said to be common in malignant cells (Kemp, 1930; Levine, 1931; Ludford, 1942). The chromosome numbers of the cells of a single tumour may vary greatly (Ludford, 1930, 1942). Binucleate and multinucleate tumour cells have been reported (Levine, 1931; Koller, 1943) and in mouse epidermis during early carcinogenesis (Pullinger, 1940).

Similar aberrations occur in non-neoplastic cells. The mitotic abnormalities observed in malignant cells can be produced in normal cells by a variety of non-carcinogenic and carcinogenic substances (Ludford, 1953). However, strains of malignant cells cultivated *in vitro* (Ludford, 1942) and many tumours *in vivo* are characterized by a high incidence of abnormalities. The structural abnormalities of malignant cells may be regarded as consequences of the malignant state (e.g. Powell, 1946) or as being directly related to the initiation and retention of malignancy. For example, disturbances in the euchromatin-heterochromatin balance suggested by Darlington and Thomas (1941) and Koller (1943) as the initiating cause of malignancy could result from derangements of the spindle and subsequent variations in chromosome number and arrangement.

In view of the debatable significance of cytological abnormalities in relation to the cancer problem it is important to distinguish genuine from false aberrations. During studies upon Ehrlich carcinoma and Sarcoma 37 mouse ascites tumour cells cultivated *in vitro* an apparent membrane-like structure enclosing the dividing nucleus was seen in a small proportion of cells. This structure was found to be associated with a particular sequence of degenerative changes in freshly explanted cells. Seen as an isolated phenomenon this " membrane " might be interpreted

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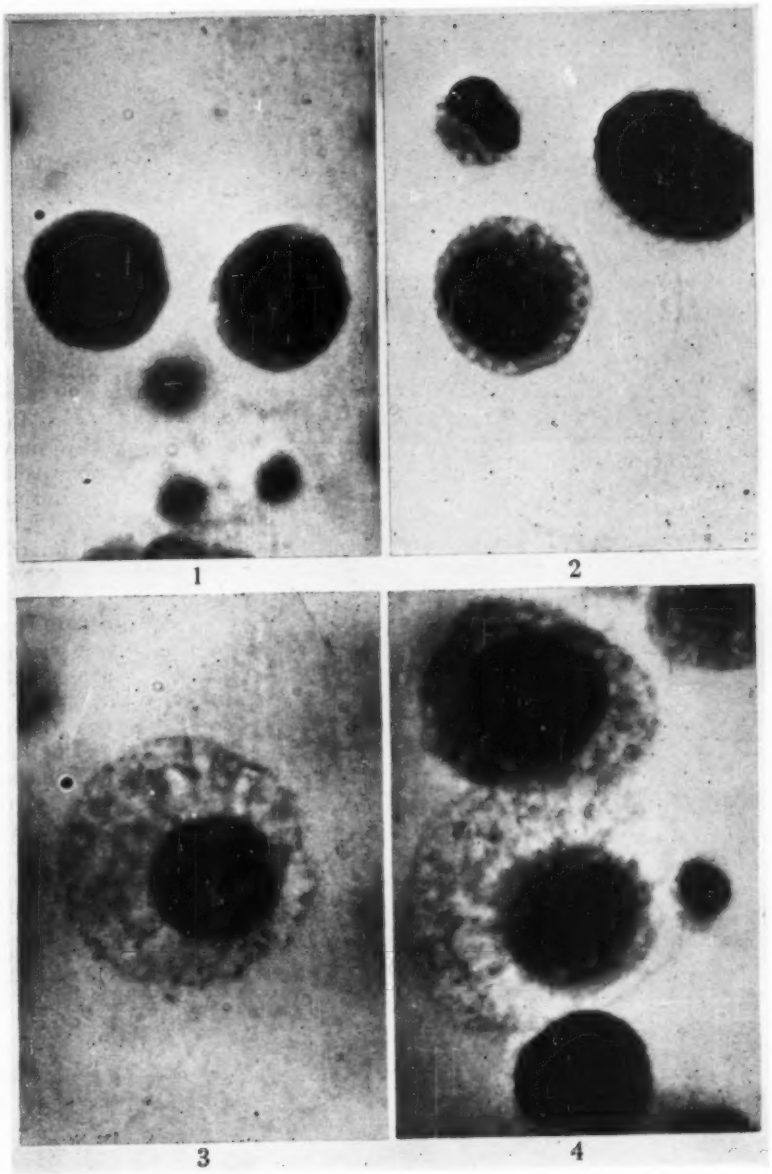
EXPLANATIONS OF PLATE

Photomicrographs of normal and degenerating Ehrlich carcinoma ascites cells in plasma cultures.

FIG. 1.—Resting cell, normal.
FIG. 2.—Resting cell, early stage of degeneration.
FIG. 3.—Resting cell, later stage of degeneration.
FIG. 4.—Cell in prophase, degenerated.
FIG. 5.—Cells in metaphase, normal and degenerated.
FIG. 6.—Cell in metaphase, degenerated.
FIG. 7.—Cell in anaphase, normal.
FIG. 8.—Cell in anaphase, degenerated.



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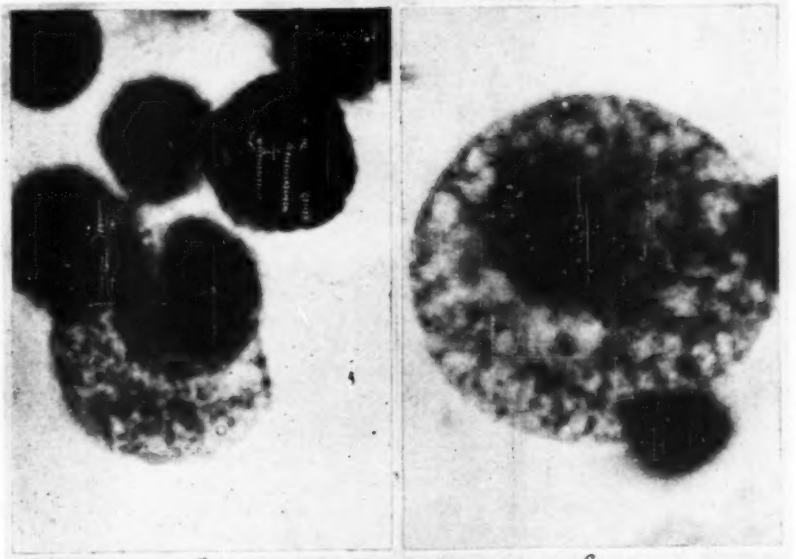

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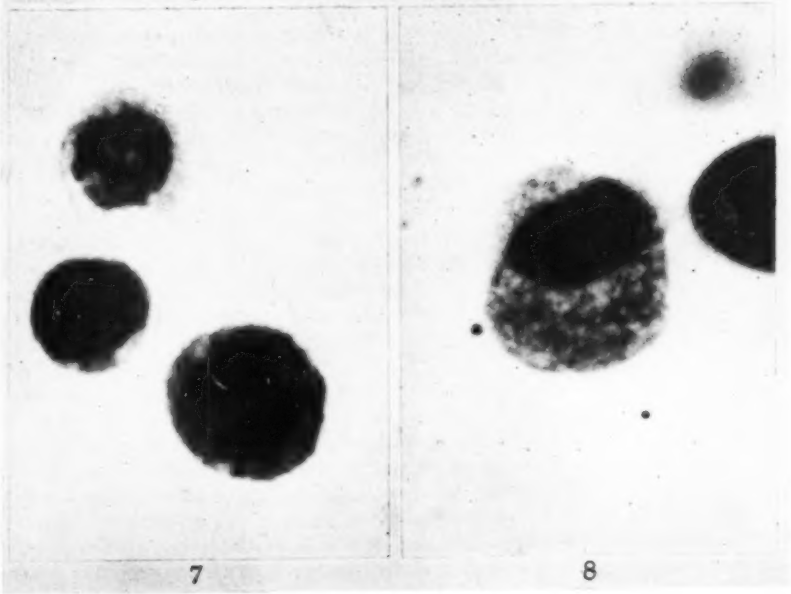

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as a veridical cellular structure and given an unwarranted significance. For this reason a brief account is given of its appearance and occurrence in the tumour cells studied.

MATERIAL AND METHODS

False membranes were found in a small proportion of cells of both strains which had been explanted in plasma-embryo extract coagula in coverslip cultures. Freshly collected ascites tumour fluid was diluted with from 9 to 99 parts of a mixture of equal parts of heparinized fowl plasma and ascites tumour plasma. The latter was prepared almost immediately before use by centrifuging fresh ascitic fluid. Homologous ascitic plasma was used for each strain of tumour. Equal volumes of the diluted ascitic fluid containing the tumour cells and either chick or mouse embryo extract prepared with 10 per cent hypotonic Earle's buffered saline solution were mixed and spread in a thin coagulum to prepare the coverslip culture. The cultures were fixed in Heidenhain's Susa mixture and stained with dilute Harris' haematoxylin. The development of false nuclear membranes was readily induced by leaving thinly populated cultures exposed in covered petri dishes at laboratory temperatures for 30 or more minutes before fixation.

OBSERVATIONS

Four main classes of degenerating ascites tumour cells were found in the cultures. Three of these were distinguished by marked hydropic vacuolation, simple autolytic changes, and changes due primarily to mechanical stress in the coagula, respectively.

Psuedo-membranes were associated with a fourth type of degeneration which was initially distinguished by progressive alterations in the structure of the cytoplasm. In contradistinction to the homogeneous cytoplasm of a healthy cell with its few small fat droplets (Fig. 1), degenerating cytoplasm developed very numerous small vacuoles and appeared reticulated (Fig. 2, 3). The width of the cytoplasm was greatly increased in comparison with the typical narrow zone in healthy cells. With further degeneration the vacuoles enlarged and the cytoplasm became more distended. Finally, the reticulated strands of protoplasm broke into discrete granules and the vacuoles coalesced.

During early stages of this form of degeneration the nuclei retained a normal structure, although it was optically sharper than in healthy cells, and affected cells could be considered viable. Later, the nuclei became pyknotic or lytic. In non-dividing cells vacuolated cytoplasm occupied the cell from the pellicle to the nuclear membrane.

Dividing tumour cells in which the nuclear membranes had dissolved reacted to the early cytoplasmic changes so as to suggest the presence of a persistent nuclear membrane during metaphase, anaphase and telophase stages (cf. Fig. 5–8). In such cells the nuclear region appeared relatively normal. It was occupied by chromosomes embedded in mitotic spindle substance which appeared structurally normal, sometimes showed axial striae and lacked vacuoles. The demarcation between spindle substance and the vacuolated cytoplasm was well delimited but not as sharply defined as a true nuclear membrane. Prophase nuclei of cells in early stages of degeneration were sometimes surrounded by a narrow zone of unvacuolated protoplasm, itself enclosed in a much wider zone of vacuolated cytoplasm (Fig. 4).

In more degenerated cells the chromatin condensed and general lysis supervened. On the other hand, in the early stages of degeneration many dividing cells with psuedo-membranes could be considered viable by the criterion of normal nuclear structure even though the cytoplasm had already become finely vacuolated and extended. The degeneration of the cytoplasm preceded that of the nuclei as in non-dividing cells. Apparently viable cells with psuedo-membranes were most abundant in cultures fixed shortly after preparation and before incubation. Cell death was obvious in such degenerating cells of cultures incubated overnight. The phenomenon of psuedo-membranes was most evident in cultures prepared with thin films of coagulum in which the cells were widely separated.

DISCUSSION

Apparent nuclear membranes occurring in dividing tumour cells have been reported by other workers. Hsu (1954) observed occasional dividing cells with a membrane-like structure enclosing the mitotic spindle and chromosomes or prophase nuclei, in cultures of the HeLa carcinoma cells. He emphasized that the " membrane " was not a true nuclear membrane but did not suggest a definite explanation of its occurrence. His illustrations of the " membrane " in cells in metaphase, anaphase and late cleavage stages show the co-existence with the " membrane " of coarsely reticulated cytoplasm and clear blebs under the cell pellicle. Similar " nuclear membranes " have been reported in cultivated chick embryo cells (Stillwell, 1952) and ascites tumour cells (Levan and Hauschka, 1953).

Calcutt and Yetts (1954) observed a similar " membrane " in some dividing cells of a transplantable mouse sarcoma. It was found in sections of tumours treated with various fixatives and stains and also in fresh smears of tumour observed by phase contrast microscopy. They pointed out that the " membrane " did not appear to be a fixation artefact and suggested it was either a persistent true nuclear membrane or arose *de novo* during mitosis and afterwards disappeared. The latter phenomenon could, they further suggested, result from the localized precipitation of interacting nuclear and cytoplasmic proteins during mitosis.

The observations made during the present work indicate that the development of a false nuclear membrane during mitosis is a consequence and an indication of cellular degeneration. The changes in the degenerating cytoplasm were similar in resting and dividing cells. The presence of the psuedo-membranes in the latter is due to the clear distinction between the vacuolated cytoplasm and the unvacuolated spindle substance. The mitotic spindle is gelated (Chambers, 1917) and the interconnections of its component protein microfibrils may account for its resistance being greater than that of the cytoplasmic matrix to dissociation and vacuolation. The mitotic spindle has been stated (Conklin, 1924 ; White, 1954) to be formed partly from nuclear and partly from cytoplasmic material. The latter source could explain the occurrence of psuedo-membranes around prophase nuclei. The development of these structures is associated with a flattening of the injured cells in the plane of the coverslip. This flattening is promoted by the partial desiccation of a thin plasma coagulum. The mitotic cells under observation were presumably dividing at the time of explantation. This particular type of cellular degeneration resulted from the experimental manipulation of the cells.

The true nature of psuedo-membranes around dividing nuclei is evident only in the context of the full sequence of degenerative changes. Seen as occasional

isolated phenomena, psuedo-membranes could readily be misinterpreted as veridical cytological abnormalities in tumour cells.

SUMMARY

The occurrence and appearance of apparent membranes enclosing nuclei of some dividing cells of Ehrlich carcinoma and Sarcoma 37 ascites tumours cultured *in vitro* are described. It is suggested that these structures are produced in cells undergoing a particular type of degeneration and are due to the clear distinction between vacuolated cytoplasm and unvacuolated spindle substance.

My thanks are due to Mr. G. A. Butcher for his technical assistance with this work.

The expenses of this research were defrayed from a block grant by the British Empire Cancer Campaign.

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INDUCTION OF MAMMOGENESIS BY PROLACTIN AND BY HUMAN URINE

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Received for publication February 1, 1957

HIGHLY significant growth responses in the rudimentary mammary glands of intact weanling male mice of the S.A.S.3 strain have been induced by pituitary prolactin when administered twice daily for five days by subcutaneous injection (Hadfield and Young, 1956). Compared with the responses produced by oestrone in the same strain at various dose levels over the same short period of injection, the mammae of prolactin-treated weanling males showed a significantly greater degree of glandular differentiation with the production of easily recognisable acini. Both hormones induced rapid development of the mammary ducts and ductules.

During the last two years, and until a few months ago, we have injected, twice daily for five days, a large number of specimens of human female urine into male weanlings of the S.A.S.3 strain, no other strain being used. Unmistakable mammary growth reactions have been obtained with the majority of urine specimens and these show the same degree of duct development and glandular differentiation which characterises the weanling mammae reacting to prolactin. Similar reactions to urine were obtained when specimens from normal pre-menopausal women in the post-ovulatory phase of menstruation were used, and significant responses were induced by the urine of 57 per cent of post-menopausal women.

Some months ago the mammary glands of the weanling males of the S.A.S.3 strain showed a precipitate fall in their ability to react both to prolactin and to human female urine (this has since returned). An extensive search was therefore made for another strain whose weanling males are sensitive to prolactin and urine. This has proved successful but the enquiry revealed the disconcerting fact that in the large majority of common strains of laboratory mice the mammary glands of the weanling males are quite unresponsive to prolactin and that this characteristic always appears to be associated with complete mammary insensitivity to human female urine. This observation explains the failure of several workers to confirm our published experiments which demonstrated the mammogenic property of human female urine when the " sensitive " mamma of S.A.S.3 weanling males was used as the " indicator ".

Other relevant facts have emerged from this enquiry. They throw some light on the nature of the mammotrophic hormone present in the urine of normal women and the present paper is a record of experiments dealing with these problems.

METHODS

For each experiment five or more weanling males, aged 25 days on the day of the first injection, were used. They were given 0·2 ml. twice a day for a period

of five days, of the untreated urine of normal pre-menopausal women in the second half of the menstrual cycle, or 0·1 ml. of oestrone or 0·1 ml. of prolactin in varying concentrations (see below). The intensity of the mammary response was measured by counting the end-bulbs or clubs in each gland. The yield of mammary glands per weanling was 5 or 6, so each batch of five animals produced about 27 glands. The average number of clubs per gland is taken to be a reasonably accurate measure of growth response. The average number of clubs per gland in untreated male weanlings in the strains examined was less than 1 in the majority and only fractionally over 1 in the remaining few.

TABLE I.—*Strains of Mice Investigated*

Strain	Source of supply
Non-inbred	
Schneider	Medical Research Council.
Parkes	" " "
Stock	Chester Beatty Research Institute.
Grey	Laboratory Animals Bureau, Medical Research Council.
Swiss	Evans.
Schneider	Tuck.
No. 1	"
No. 2	"
Tyler's originals	"
S.A.S.3	Scientific Animal Service.
S.A.S.4 (Tyler's originals)	" " "
In-bred :	
C_3H	Imperial Cancer Research Fund, Mill Hill Laboratories.
$C_{57}Bl$	" " " "
C −	Chester Beatty Research Institute.
C +	" " " "
C_3H	" " " "
Hairless	" " " "
$C_{57}Bl$	" " " "
CBA	" " " "
Strong A	Laboratory Animals Bureau, Medical Research Council.
AKR	"
BALB	"
$C_{57}Br$	"
CE	"
C_3H	Medical Research Council.
Strong A	Guy's Hospital Medical School.
BALB	" " " "
A_2G	Glaxo Laboratories.
GFF	" "
JU	Institute of Animal Genetics, University of Edinburgh.
RIII	"
F1 crosses from in-bred strains :	
AKR × CE	Laboratory Animals Bureau, Medical Research Council.
AKR × BALB	"
DBA × A	"
CE × A	"
CE × BALB	"
A × BALB	"

Urines proved to be potent failed to produce a significant mammary growth response in any of these 37 strains. Twelve of these strains, including the reacting S.A.S.3 and A_2G, were further examined for their ability to react to oestrone and prolactin. The strains were selected because of their popularity as experimental animals and their ready availability.

RESULTS

Thirty-seven strains, as shown in Table I, have been examined.

Response to urine.—The mammae of A_2G mice were found to react vigorously to normal pre-menopausal urine and as the S.A.S.3 mice were found to have regained their previous sensitivity, a batch of weanlings from each of these strains was included in every experiment as controls. This indicated whether the urine was active or not and gave a measure of its potency.

Response to oestrone.—Using oestrone (B.D.H.) dissolved in 50 per cent aqueous propylene glycol, the optimum dose level was determined over a total dose range of 0·001 to 1·0 μg. given in ten injections over a period of five days. The latter concentration was selected as giving a typical and vigorous response and oestrone at this level was therefore administered to groups of animals from each of the twelve strains, including A_2G and S.A.S.3. Unmistakable mammary growth responses were induced in all strains without exception.

Response to prolactin.—Prolactin (Squibb) dissolved in distilled water containing 0·5 per cent phenol was administered to A_2G and S.A.S.3 mice over a total dose range of 0·004 to 40·0 international units (by pigeon crop assay) given twice daily for a period of five days. Forty i.u. was selected as an appropriate total dose. This was administered to animals from each of the 12 strains used for the oestrone response, again including A_2G and S.A.S.3. Excluding the two latter, prolactin failed to elicit any significant mammary response in the remaining 10 strains. In S.A.S.3 and A_2G weanlings the reactions were unmistakable but rather less vigorous in the S.A.S.3 animals. The results are summarised in Table II in which the growth response is recorded as follows:

Average number of clubs per gland	Designation
Below 3·5	−
Between 3·5 and 4·5	±
Between 4·5 and 7·0	+
Over 7·0	++

CONCLUSIONS

1. The mammary growth responses to human urines known to possess mammotrophic potency have been investigated in the weanling males of 37 strains of laboratory mice. In two strains only—S.A.S.3 and A_2G—was a significant growth response induced.

2. Twelve of these strains, including those possessing mammary sensitivity to human pre-menopausal female urine, responded satisfactorily to oestrone; the reaction was confined to the duct system; there was little or no glandular acinisation.

3. Two strains only—S.A.S.3 and A_2G—responded to prolactin. In these the response was vigorous and unmistakable and early acinisation was common.

4. Strains responding to prolactin also responded satisfactorily to human female urine.

5. Strains failing to respond to prolactin failed to respond to human female urine.

6. These results support the contention that the mammotrophic agent present in human pre-menopausal female urine is prolactin.

TABLE II.—*Mammary Growth Response in Various Strains of Mice Induced by Oestrone, Prolactin and Human Female Urine from Normal Pre-menopausal Women: Injections Twice a Day for Five Days.*

Strain of mice	Source of supply	Degree of response induced: Oestrone: Total dose 1·0 µg.	Prolactin: Total dose 40 i.u.	Urine: Total dose 2·0 ml.
Non-inbred:				
S.A.S.3	Scientific Animal Service	++	+	+
S.A.S.4	" " "	++	−	−
Schneider	Medical Research Council	++	−	−
Parkes	" " "	++	−	−
Grey	Laboratory Animals Bureau, Medical Research Council	++	−	−
In-bred:				
A_2G	Glaxo Laboratories	++	++	++
A	Laboratory Animals Bureau, Medical Research Council	0	−	−
A	Atomic Energy Research Establishment, Harwell	+	−	0
BALB	Laboratory Animals Bureau, Medical Research Council	++	−	−
C_3H	Chester Beatty Research Institute	++	−	−
$C_{57}Bl$	" " " "	++	−	−
F1 crosses from inbred strains:				
CE × BALB	Laboratory Animals Bureau, Medical Research Council	++	−	−
AKR × BALB	" "	++	−	−

0 = Not investigated.

7. Weanling males of the A_2G strain of mice are suitable for detection of the mammotrophic agent present in human female urine. Weanlings of the large majority of other strains are quite unsuitable.

We are indebted for supplies of animals to Dr. J. Craigie, Dr. W. F. J. Cuthbertson, Dr. D. S. Falconer, Dr. P. A. Gorer and Professor A. Haddow. Dr. W. S. Lane Petter of the Laboratory Animals Bureau, Medical Research Council, has not only arranged for supplies of mice but has given valuable help and advice. Messrs. E. R. Squibb and Sons kindly supplied the prolactin used in these experiments.

REFERENCE

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ENZYME ACTIVITY IN RELATION TO CANCER

The Urinary β-Glucuronidase Activity of Patients Suffering from Malignant Disease

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Received for publication November 3, 1956

A connection between the process of cell proliferation and the increase in tissue β-glucuronidase has been suggested by Kerr and Levvy (1947) and Levvy, Kerr and Campbell (1948). Fishman (1947) and McDonald and Odell (1947; Odell and McDonald, 1948) have investigated the changes of serum β-glucuronidase activity in human pregnancy and Fishman (1947), Fishman and Anlyan (1947*a*, 1947*b*) and Fishman, Anlyan and Gordon (1947) have shown that β-glucuronidase is present in enhanced amounts in human and animal cancer tissues.

The β-glucuronidase activity of body fluids has been investigated by several workers in the hope of developing a method of diagnosis or prognosis of cancer. The β-glucuronidase activity of blood serum, ascitic and pleural fluids (Fishman, Markus, Page, Pfeiffer and Homburger, 1950), spinal and ventricular fluid (Anlyan and Starr, 1952) and of vaginal fluids (Fishman, Kasdon and Homburger, 1950) has been estimated in patients suffering from various forms of cancer especially cancer of the breast and cervix.

Boyland, Wallace and Williams, (1955*a*, 1955*b*) have shown that cancer of the bladder is associated with high β-glucuronidase levels in the urine, serum and bladder tissue. The present work deals with the urinary β-glucuronidase values of cases of cancer of sites other than the bladder. All the patients were in hospital, on balanced diets, and all the specimens were collected before any operative procedure was undertaken.

EXPERIMENTAL

The urine was collected in 2-litre vessels containing thymol dissolved in benzene (10 ml. of 20 per cent solution). Individual 24-hour specimens were measured and their pH and specific gravity determined. Samples were centrifuged at 450 g. for 15 minutes and the supernatant examined generally within 24 hours of collection being completed.

The estimation of β-glucuronidase activity in urine

The method used was essentially that of Talalay, Fishman and Huggins (1946) which has been modified for routine analysis (Boyland, Wallace and Williams, 1955). The method has been further modified by increasing the strength of the acetate buffer (pH 4·5) since, especially in the case of grossly infected urine, the urine is sometimes alkaline and therefore greater buffering power is necessary

in order to maintain constant pH. Table I shows the effect of various buffer concentrations on the pH of five typical urine samples. The system previously used (*b*) does not always bring the pH of the urine mixture within the required limits of 4·3–4·8 (Boyland, Wallace and Williams, 1955) but, by using 0·2 M acetate buffer (*c*) the required conditions can be established. Under these conditions neither the original alkaline buffer (*d*) nor a buffer of twice its strength (*e*) is sufficiently strong to give a final pH within the range 10·0–10·5. The system (*g*) was finally selected as the one giving the most constant pH conditions.

TABLE I.—*The Effect of Buffer Concentration on the pH of the Test Solution*

Specimen No.	(pH) 1	2	3	4	5
a. Urine alone	6·30	5·20	5·70	7·00	5·95
b. Urine + substrate + 0·1 M acetate buffer	4·85	4·65	5·00	5·25	4·85
c. Urine + substrate + 0·2 M acetate buffer	4·50	4·55	4·75	4·80	46·5
d. Urine + substrate + 0·2 M acetate buffer + 0·4 M glycine buffer	9·50	8·70	8·40	9·00	8·90
e. Urine + substrate + 0·2 M acetate buffer + 0·8 M glycine buffer	10·0	9·70	9·50	9·75	9·70
f. Urine + substrate + 0·2 M acetate buffer + 10% $Na_2CO_3 10H_2O$	10·1	10·0	9·7	10·0	9·95
g. Urine + substrate + 0·2 M acetate buffer + 10% Na_2CO_3 (anhyd.)	10·3	10·30	10·10	10·30	10·25

The stability of the phenolphthalein colour at pH values above 9·5 has been investigated as this colour tends to fade at high pH. The colour produced by a series of standard enzyme solutions was investigated at pH values between 9·5 and 11·5 and found to be maximal between pH 10·2 and 10·6. The phenolphthalein colour is stable for 2 hours within the pH range 10–10·5 so the system (*g*) (Table I) is satisfactory and has been used throughout this work.

Urine (1 ml.) acetate buffer (1 ml.) (0·2 M, pH 4·5) and substrate solution (1 ml. 0·05 per cent) were incubated in stoppered tubes for 18 hours at 37° in a water bath, urine (1 ml.) was added to the blank and sodium carbonate solution (1 ml. 10 per cent Na_2CO_3) was added to each. The tubes were centrifuged and the duplicates read against the blank on a Unicam S.P. 500 spectrophotometer at 550 mμ. The activity was expressed in units, 1 unit liberating 1 μg. of phenolphthalein per hour at 37°.

Owing to the modification of the method of estimation, the units are different from those used previously (Boyland, Wallace and Williams, 1955), so that the normal range (Table II) is now taken as 0·05–1·2 units/ml. of urine. Values in the pH range of 5·0–7·0 are regarded as normal and deviation from this range is an indication of infection of the urine. The specific gravity of the urine provides a check on the complete collection of the specimen so that both pH and specific gravity have been quoted for each specimen.

It has been suggested (Boyland *et al.*, 1955; Boyland and Williams, 1956) that both the metabolism of tryptophan and the β-glucuronidase activity of the urine play a part in the production or maintenance of bladder tumours. The amount of tryptophan in the diet might affect the apparent urinary β-glucuronidase activity, because tryptophan metabolites conjugated with glucuronic acid might act as competitive inhibitors. A number of specimens have been examined after dosing the patients with 2 g. of *L*-tryptophan. Such specimens are indicated in the tables by the presence of the letter T after the figure for urine volume. All specific gravities are given in direct urinometer readings, thus a figure of 10 indicates a specific gravity of 1·010.

TABLE II.—*Normal Subjects*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	M.	30	3250	6·5	—	0·05
2	F.	—	1890	6·1	11	0·14
3	M.	29	3500T	6·5	—	0·17
4	F.	63	1300	7·0	—	0·32
5	M.	68	1300	5·8	—	0·35
6	M.	26	2560	6·4	12	0·36
7	M.	72	1910	6·1	10	0·36
8	F.	—	2770	7·0	22	0·44
9	M.	48	2370T	6·2	06	0·59
10	M.	68	1450	5·5	—	0·60
11	M.	65	1670	7·0	06	0·60
12	M.	58	1440	5·5	14	0·74
13	M.	31	1550	6·5	—	0·80
14	F.	21	1150	7·0	15	0·81
15	M.	59	1830	6·4	08	0·87
16	M.	50	5500	5·3	12	0·89
17	M.	51	1830T	6·8	20	0·90
18	F.	—	1100T	7·0	15	0·93
19	M.	71	2520	5·6	10	1·0
20	M.	31	1800T	6·3	—	1·1
21	M.	60	2120T	5·8	09	1·2

Patients suffering from cancer of the alimentary tract

The urinary β-glucuronidase of patients suffering from cancer of the alimentary tract varies with the site of the tumour. The β-glucuronidase values of 5 patients out of 8 suffering from cancer of the stomach lie within the normal range and 3 are high (Table III). The mean value is 1·2 units which is the upper figure of the normal range. Of 6 cases of argentaffinoma (Table IV) only one has an abnormally high activity and the average activity is 1·2. The β-glucuronidase actitity of 5 out of 7 patients with cancer of the oesophagus (Table V) have values within the normal limits but the average enzyme value is 1·5 units. All 5 of the cases of cancer of the colon (Table VI*a*) have low β-glucuronidase activity with an average activity of 0·52 units and the urine volumes in these cases are also low. The activities in the urines of five patients with cancer of the rectum Table VI*b*) are on or above the normal limit with an average value of 1·4 units.

TABLE III.—*Patients Suffering From Cancer of the Stomach*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	M.	48	1560	5·7	10	0·45
2	M.	42	2380	6·1	03	0·70
			2450T	6·4	04	0·50
3	M.	—	1100	7·0	20	0·60
4	M.	56	1080	5·8	—	0·84
5	M.	36	1470	5·8	12	1·3
			1930	6·7	12	1·6
			1600	6·0	09	1·1
6	M.	39	900	5·6	—	1·5
7	M.	65	1240	5·5	15	1·7
			1120	5·6	12	1·8
8	M.	54	1460	6·2	19	2·8
			1290	6·2	14	2·9
			2040	6·8	10	2·3

TABLE IV.—*Patients Suffering from Argentaffinoma*

Case No.	Sex.	Age	Urine Vol (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	M.	—	1800	6·4	—	0·7
2	M.	54	1900	7·9	—	0·8
3	M.	—	—	7·9	—	0·9
4	M.	70	2020	5·6	11	1·1
			1590	5·8	12	0·8
			2320T	5·8	09	0·9
			1700T	5·5	11	0·7
5	M.	65	735	6·4	—	1·0
6	M.	39	1025	7·3	—	3·2

TABLE V.—*Patients Suffering from Cancer of the Oesophagus*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	F.	68	1890	4·8	—	0·30
2	M.	45	2050	5·8	—	0·80
3	M.	61	3320	5·8	08	1·1
5	M.	67	2220	5·8	09	1·0
			1640	5·8	08	1·4
5	M.	39	890	5·8	12	1·2
6	M.	51	1530T	5·5	12	1·3
7	M.	61	370	5·5	24	4·2
			450	5·5	26	4·2

TABLE VI.—*Patients Suffering from Cancer of the Colon or Rectum*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
			(a) *Colon*			
1	M.	66	1320	5·6	—	0·27
2	F.	53	890	6·4	12	0·46
			1130	6·4	08	0·28
3	M.	47	1800	5·4	—	0·54
4	F.	58	1550	5·2	—	0·65
5	M.	68	700	5·2	09	0·77
			(b) *Rectum*			
1	M.	55	1250	6·0	08	1·1
2	M.	72	1500	5·5	10	1·1
			1800	5·6	10	1·2
3	M.	65	1640	6·0	15	1·5
			1310	7·0	12	1·4
4	M.	45	2100	6·1	15	1·3
			1340	6·4	20	1·7
			2370	5·5	06	1·9
5	M.	72	1450	5·5	10	1·6

Patients suffering from cancer of the larynx

Of the 20 patients suffering with cancer of the vocal cord (Table VII) only 4 have β-glucuronidase activities within the normal range, with a mean 1·7 units. Of 11 patients suffering from cancer of the larynx but not cancer of the vocal cord (Table VIII), 5 had values above the range of healthy subjects, the mean value being 1·6 units.

TABLE VII.—*Patients Suffering from Cancer of the Larynx*

(a) *Cancer of the Vocal Cord*

Case No.	Sex	Age	Urine Vol (ml.)	pH	S.G.	β-glucuronidase units/ml.
1	M.	50	2750	5·5	10	0·60
2	M.	52	1210T	5·5	10	0·60
			1100	5·5	08	0·70
3	M.	84	2120	5·5	11	1·2
			2290	5·5	12	1·0
4	M.	68	2630	6·7	0·4	0·8
			2380T	6·8	0·5	1·6
5	M.	63	1330	6·0	14	1·5
			2590T	7·2	07	1·0
6	M.	61	2300	6·7	10	1·5
			1490T	5·5	10	1·0
7	M.	60	1010	—	—	1·4
8	F.	—	650	5·4	—	1·5
9	M.	58	1770	5·8	21	1·5
			2060	5·9	08	1·5
10	M.	67	710	7·0	18	1·6
			1330T	6·4	10	1·5
11	M.	35	1700	5·5	19	1·7
			1910T	5·6	12	1·3
12	M.	67	1130	5·5	14	1·7
13	M.	60	710	6·0	29	1·5
			540T	5·5	15	2·3
14	M.	64	1080T	5·8	17	1·8
15	M.	61	2100	6·1	11	1·7
			2320T	5·5	10	1·6
			660	5·8	15	2·5
16	M.	38	910T	5·8	15	2·4
			710	7·0	18	1·6
17	F.	61	2500	5·8	—	2·9
			1600	6·4	11	1·1
18	M.	55	740	5·6	23	2·5
			850T	5·5	28	3·1
19	M.	83	1270	7·5	10	3·4
			1260	7·3	13	3·7
20	M.	73	890	6·1	18	4·1
			900T	5·6	14	3·2

TABLE VIII.—*Patients Suffering from Cancer of the Larynx*

(b) *Cancer of Sites other than Vocal Cord*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	M.	69	2350T	5·5	10	0·6
2	M.	61	2340T	6·1	06	0·7
3	M.	57	2660	7·0	06	1·0
			2520T	7·0	14	0·7
4	M.	56	2550T	6·7	10	1·0
5	F.	84	940	6·0	10	1·0
			1530T	5·8	05	1·2
6	M.	39	890	5·8	12	1·2
7	M.	51	1530T	5·5	12	1·3
8	M.	64	2590T	6·4	09	1·6
9	M.	62	1720T	5·8	07	1·7
10	M.	54	1370T	6·9	18	2·5
11	M.	57	860T	7·0	15	2·7

Patients suffering from cancer of the bronchus

Of 11 patients suffering from cancer of the bronchus (Table IX) 5 cases have a β-glucuronidase activity within the normal range and the mean is 1·8 units.

TABLE IX.—*Patients Suffering from Cancer of the Bronchus*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	F.	72	1700	6·0	10	0·50
			1090	5·5	14	0·90
2	M.	49	2270	6·1	16	0·60
			2800T	7·0	06	1·1
3	M.	75	500	—	—	1·0
4	M.	57	1720	5·5	12	1·0
			860	5·8	13	1·2
5	M.	62	720	6·8	12	0·8
			1200	6·0	—	1·5
6	M.	—	980	6·1	10	1·6
7	M.	66	1230	6·4	21	1·7
			1980	6·4	24	1·8
8	M.	62	720	6·8	12	0·8
			1200	6·0	—	1·5
9	M.	69	1400T	6·4	15	2·4
10	M.	63	1400	7·0	14	2·4
11	M.	56	870	6·8	14	4·8
			1370	6·8	14	4·9

Patients suffering from malignant blood diseases

The urinary β-glucuronidase activity of 8 out of 10 patients suffering from leukaemia (Table X*a*) was above the normal range with an average activity of 1·8 units. Cases 9 and 10 had pyrexia at the time of collection. Of 5 patients suffering from Hodgkin's disease (Table X*b*) 4 had activity above the normal range and the average value is 2·5 units.

TABLE X.—*Patients Suffering from Malignant Blood Diseases*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
			(a) *Leukaemia*			
1	M.	44	1500	6·0	—	1·5
2	M.	24	2200	5·7	—	1·2
3	M.	50	2500	6·7	09	1·2
			2100	6·7	11	0·98
			2500	5·8	67	1·9
4	F.	71	1850	7·0	10	1·7
			1860T	7·0	09	1·2
5	F.	76	1760	6·2	—	1·5
6	F.	44	1500	6·0	—	1·5
7	M.	70	1440	6·7	12	1·9
8	F.	16	1630	7·0	20	2·7
			1410T	7·4	24	2·4
9	F.	26	1680	5·8	09	3·0
10	M.	38	2620	7·0	10	3·4
			(b) *Hodgkins Disease*			
1	M.	34	1890	6·4	07	0·50
			1890	6·0	08	0·70
2	M.	48	720	5·8	20	1·3
			1630	6·4	15	2·7
3	M.	42	2800	5·8	05	3·0
			1800	6·1	12	2·8
			1200	6·0	10	2·7
4	F.	50	420T	5·8	28	3·2
5	M.	63	1440	5·5	20	3·6
			710	7·0	—	3·8

Patients suffering from cancer of the breast

Of 15 patients suffering from cancer of the breast (Table XI), 8 are above the normal range of activity. Case 5 is a male with cancer of the breast. The average β-glucuronidase activity was 1·5 units.

TABLE XI.—*Patients Suffering from Cancer of the Breast*

Case No.	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronisdase Units/ml.
1	55	1250	6·7	09	0·3
2	52	2500	5·8	—	0·8
3	47	1820	5·5	—	1·0
4	48	2400	6·9	10	1·2
5	42	1910T	5·5	12	1·2
6	55	1200	5·5	—	1·2
7	55	1750	6·2	—	1·2
8	62	1570	4·8	—	1·5
9	53	1820	7·0	08	1·6
10	61	2000	6·5	—	1·9
		1860	9·3	13	1·3
11	48	1140	6·0	18	2·2
		1800T	5·8	14	1·3
12	38	1375	6·5	—	1·8
13	78	2020	—	—	2·2
14	54	960	4·5	—	2·5
15	63	1200	5·6	14	2·6
		1030T	5·6	12	2·5

Patients suffering from cancer of the testicle

Of 15 patients suffering from cancer of the testicle (Table XII) 10 have β-glucuronidase values above the normal range. The average β-glucuronidase value is 1·5 units.

TABLE XII.—*Patients Suffering from Cancer of the Testicle*

Case No.	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	29	1800	—	—	0·80
2	39	1400	5·4	—	0·85
3	45	610	5·8	24	1·0
4	35	1750	6·5	—	1·0
5	55	1830	8·5	11	1·2
6	37	2190	7·6	14	0·8
		2000T	7·0	12	1·6
7	45	2100	6·1	15	1·3
		1340	6·4	20	1·7
		2370	5·5	06	1·0
8	18	1500	5·5	—	1·5
		900	6·0	—	1·3
9	31	2100	7·0	—	1·4
		2100	—	—	1·4
		2200	6·5	—	1·6
10	31	2100	7·0	—	1·4
11	18	1500	5·4	—	1·5
12	30	1500	5·5	—	2·5
		2550	5·8	—	1·2
13	68	1270	5·5	10	2·5
		1330	5·5	10	2·2
		1730	5·5	12	2·0
14	39	1250	5·9	18	1·8
		450T	5·5	12	4·0
15	29	1080	—	16	4·2
		940	—	18	1·7

Patients suffering from cancer of the prostate

(a) *Untreated patients.*—Of 14 patients with cancer of the prostate examined before treatment (Table XIII) one-half have values above normal range. The average β-glucuronidase activity is 1·5 units/ml.

TABLE XIII.—*Patients Suffering from Cancer of the Prostate*

(a) *Untreated Patients*

Case No.	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	75	1700	6·0	—	0·55
2	67	1700	5·5	12	0·79
3	51	1850	6·1	08	0·95
4	62	1250	5·6	—	1·0
5	58	1920	6·5	12	1·0
6	79	1060	5·2	17	1·3
		2000	5·2	08	1·0
		1500	5·2	12	1·2
7	66	1100	—	—	1·1
8	68	1720	5·8	10	0·5
		1490	6·0	08	1·0
		1100	5·8	10	1·3
		1170T	6·7	15	2·7
9	70	1910	5·7	13	1·2
		1995	5·8	14	1·3
10	57	2600	—	—	1·4
11	63	620	5·5	20	2·2
		620T	5·5	22	1·3
12	49	1290	7·0	10	2·3
		1440T	7·1	11	1·9
13	70	1230	5·5	12	2·9
		1640	5·5	09	2·6
		1940	5·8	10	3·8
14	66	1140	7·0	06	2·9

(b) *Patients treated with stilboestrol for more than two years.*—The mean β-glucuronidase activity of the urine of a group of 7 patients receiving treatment with stilboestrol for more than two years (Table XIV) is 2·3 units and all were above the normal range.

TABLE XIV.—*Patients Suffering from Cancer of the Prostate*

(b) *Patients Treated with Stilboestrol for more than Two Years*

Case No.	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	70	2070	5·5	13	1·6
		2170	7·0	13	1·5
2	69	1100	6·1	21	1·8
		1930	5·7	11	1·8
3	73	1590	6·9	20	2·0
			6·9	13	1·5
4	66	970	5·8	15	2·2
		1020	5·8	13	2·0
5	72	2580	2·3	12	—
		1570	6·4	20	2·6
6	68	1360	7·0	15	2·9
		1020	7·0	19	3·2
7	69	1650	7·0	17	2·9
		1400	7·0	15	3·3

Patients suffering from cancer of the thyroid

The results obtained from 7 patients with cancer of the thyroid (Table XV) show only two of these patients had β-glucuronidase activities greater than the upper limit of normal and in both of these cases the patients had raised temperatures at the time of collection of the specimen. The average β-glucuronidase activity of this group was 1·1 units.

TABLE XV.—*Patients Suffering from Cancer of the Thyroid*

Case No.	Sex	Age	Urine Vol. (ml.)	pH	S.G.	β-glucuronidase Units/ml.
1	F.	57	1850	5·5	—	0·35
			1800	—	—	0·36
			1900	5·8	—	0·31
2	F.	70	2500	6·0	—	0·45
			1750	6·1	—	0·58
			2300	6·0	—	0·38
3	M.	48	2120	7·0	09	0·35
			1900	6·7	11	0·60
4	F.	68	1800	5·3	—	0·50
			2050	—	—	0·52
			2010	5·6	15	0·50
5	F.	—	1500	6·1	18	0·80
			1675	6·1	22	0·50
6	F.	—	1130	6·7	19	1·8
			980	6·7	09	1·6
7	F.	68	570	7·0	15	3·4
			500	6·4	19	4·1

DISCUSSION

The β-glucuronidase excretion has been expressed as activity per unit volume of urine but in all cases the volume of the 24-hour specimens of urine have also been recorded so that total excretions of β-glucuronidase per 24 hours may be obtained. The method of estimation has been modified so that even alkaline urine specimens, previously discarded may now estimated, but in very alkaline conditions the enzyme is not stable.

The urinary β-glucuronidase activities of patients varied considerably with the site of the tumour but in no group of cases were all the results outside the normal range. This suggests that β-glucuronidase activity could not be used as a diagnostic method, although in some types of cancer such estimations might prove useful for prognostic purposes.

The average urinary β-glucuronidase activity in cancer of various sites within the alimentary tract is, in general, near the upper limit of normal but the activity in cases of cancer of the colon is low. Cancer of the thyroid also produced a decrease in urinary β-glucuronidase activity but cases of cancer of larynx, bronchus, oesophagus, prostate and testicle show an increased activity.

Cases of leukaemia and of Hodgkin's disease also exhibit an increase in urinary β-glucuronidase activity but in these cases there is generally an increase in the patient's temperature, which is known to increase β-glucuronidase excretion (Boyland and Williams, unpublished), so that the increased activity may be due partly to this cause. The connection between malignant blood disease and blood glucuronidase activity and the treatment of these patients with enzyme inhibitors is being investigated.

SUMMARY

(1) The method previously used for the estimation of urinary β-glucuronidase activity has been modified so that it may be applied to alkaline or concentrated urine.

(2) Urinary β-glucuronidase activities have been investigated in patients suffering from cancer of the alimentary tract, larynx, breast, thyroid, bronchus, testicle, and prostate and also in patients suffering from leukaemia and Hodgkin's disease.

We should like to thank Professor F. C. Ormerod of the Institute of Laryngology; Dr. L. M. Franks of the Imperial Cancer Research Fund Laboratories, Royal College of Surgeons; and Dr. M. Sandler of the Royal Free Hospital for the collection of specimens and Mr. J. W. Gorrod for technical assistance.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute for Cancer Research; Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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A MODEL SYSTEM FOR EXAMINING THE RADIOSENSITIVITY OF METABOLISING LAYERS OF CELLS

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Received for publication November 10, 1956

THOMLINSON AND GRAY (1955) examined the histological structure of some malignant human tumours, and they observed that in some squamous cell carcinomas the tumour cells lie packed close together, without any capillaries lying between them.

They suggested that :—

1. Some of these cells can get their supply of oxygen and metabolites only by diffusion, over distances which may be as great as 170 μ.
2. There must exist a steep oxygen gradient through such masses of metabolising cells.
3. Cells located far from the oxygen supply in the stroma may be relatively resistant to damage by X-rays. (Gray, Conger, Ebert, Hornsey and Scott, 1953.)

Direct proof of the survival of individual cells and their descendants from such partially, or wholly, anoxic regions is difficult to obtain by purely histological methods. It was, therefore, decided to construct a model system, in which it would be possible to identify unambiguously the cells which survived a dose of X-irradiation.

To simulate the dense areas of packed cells observed by Thomlinson and Gray (1955), tumour cells from the Ehrlich ascites tumour were centrifuged in flat-bottomed perspex tubes of 1·5 cm. internal diameter. It was found that reasonably even layers of cells could be deposited. The oxygen supply from the stroma of a tumour was simulated by placing a layer of oxygenated (human) blood on top of the tumour cells. The Ehrlich tumour cells are frequently contaminated by blood, and this was removed by the osmotic shock method described by Chance and Castor (1952) (30 seconds in an excess of distilled water followed by addition of an equal volume of double strength saline). The cells were then separated by centrifugation and resuspended in Krebs Ringer-phosphate (pH 7·2) (Umbrett, Burris and Stauffer, 1949). The red blood cells were removed from the tumour cells, because the presence of haemoglobin among the tumour cells in the packed layers would have increased the time required to reach the equilibrium condition, when the amount of oxygen entering the layer is equal to that consumed. The human blood which provided the oxygen supply for the

EXPLANATION OF PLATE.

FIG. 1.—Comparison between model system and histological section human carcinoma of bronchus.

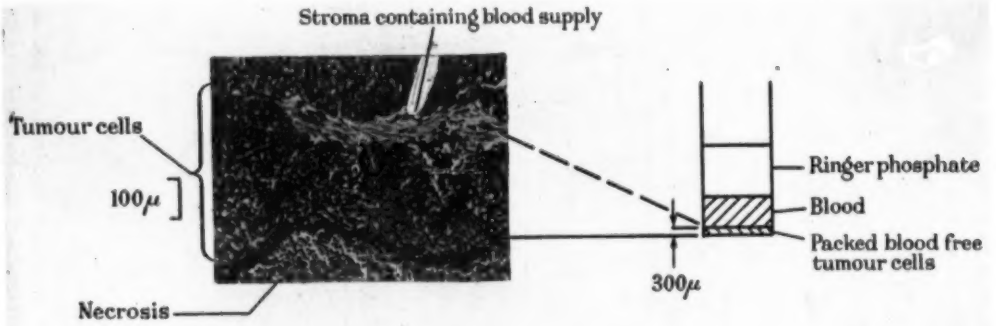
Stroma containing blood supply
Tumour cells
100μ
Necrosis
Ringer phosphate
Blood
Packed blood free tumour cells
300μ
Histology of lung tumour from Thomlinson and Gray (1955)
Model system

FIG. 1

layer was not centrifuged, but merely introduced with a syringe and needle, care being taken not to disturb the tumour cells. Tumour cells which had been freed from blood were kept at 3° C. before use.

In order to prepare thin layers of cells, the haematocrit value of a strong stock of cells was measured, the stock suspensions then being diluted with Ringer-phosphate, to give a layer of the required thickness. Since the thickness of the thinner layers was not measured directly, there may have been errors in the estimated thickness but the exact thickness of the layers is not crucial to the argument which will be developed.

The perspex tubes were irradiated from below in a small water bath. Initially the temperature was maintained at 37° C. before and during irradiations, but it was found in control unirradiated material that tumour cell survival was better at room temperature, and all the irradiations reported in this paper were carried out at around 20° C. The physical factors were 190 kV.; filter ·5 mm. Cu.+ 1 mm. Al. The dose rate was 270 r/minute.

After irradiation the contents of each tube were resuspended in the supernatant ringer-phosphate, the resulting cell-suspension being inoculated into two mice. Three perspex tubes were irradiated together, thus providing material for six mice. The strain of mice used was a heterogeneous one obtained from the Scientific Animal Service. The rate of appearance of macroscopically visible ascites tumours was used as a criterion by which to evaluate the radiosensitivity of the tumour cells.

Some preliminary experiments showed that centrifuged cell layers were more resistant to irradiation than cells suspended in aerated medium, thus confirming Patt's result with lymphocytes (Patt, Blackford and Straube, 1952).

In order to distinguish between survival of cells at different levels within the centrifuged layer, use was made of the well-known fact that heavily irradiated cells continue to metabolise for some time, although they are unable to grow. It was found that 10,000 r delivered to packed (and, therefore, probably anoxic) cells, was sufficient to prevent growth in every case, although metabolism was unaffected during the period necessary to carry out the test irradiations. Manometric measurements of oxygen uptake over a space of one hour at 37° C., kindly carried out by Dr. D. Dewey, showed no significant difference in the oxygen uptake of control and irradiated tumour cells.

Double layers of cells were prepared, in which a 250 μ thickness of heavily irradiated (H.R.) cells was spun down either above, or beneath, a 50 μ layer of unirradiated (" test ") cells. The double layer was then given the " test " dose of irradiation. Between the completion of centrifugation and the test irradiation, a period of 5 minutes was allowed for the oxygen and other metabolic gradients to be established. Tests with methylene blue (see later) suggested that the metabolic removal of oxygen was complete in a centrifuged layer within a few minutes, at room temperature.

It will be seen from Fig. 2 and 3 that at each X-ray dose level tumours appeared earlier in the animals which received an inoculum of cells from tubes in which the test cells were separated from the source of oxygen by a metabolising layer of H.R. cells. The small number of animals does not permit an accurate evaluation of the ratio of the sensitivity of the test cells in the two different positions, but comparison of the 750 r and 1500 r results in the experiment of May 16, 1956 suggests that the ratio was at least 2.

In two experiments the blood was omitted, aerated Ringer-phosphate alone providing a supply of oxygen to the packed cell layers. The result of one such experiment is shown, indicating that it is the relative position of the H. R. and test layers, not the blood as such, which causes the observed effect. This experiment does not, of course, prove that with living animals the difference between the sensitivity of cells adjacent to, or remote from, stroma, is simply due to differences in oxygen tension. Even in the model experiments there may be

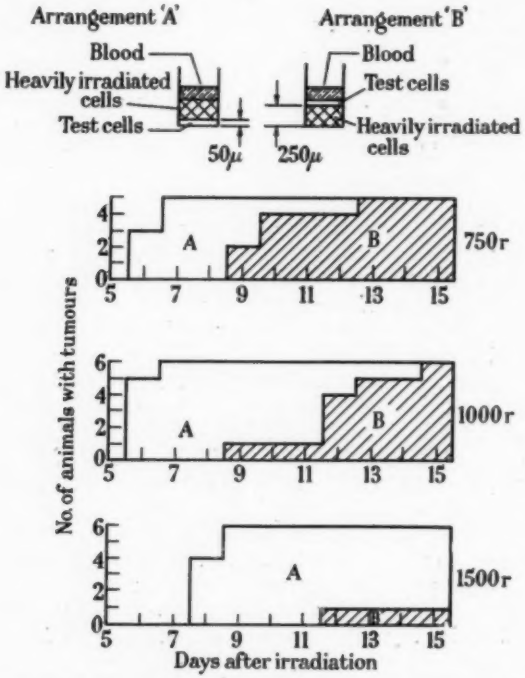

FIG. 2.—Growth of tumours from cells irradiated in arrangements A. and B. (Experiment of May 16, 1956.)

differences between the top and bottom of a metabolising layer, such as pH gradients, which may or may not be directly related to oxygen tension, and which could conceivably influence radiosensitivity. These other variables will be considered in a later paper. It is likely that oxygen depletion is a factor of major importance.

Direct evidence that an oxygen gradient is rapidly established in the tumour cell layers was obtained as follows. Methylene blue at a concentration of between M/1000 and M/10,000 was added to the blood-free tumour cells before centrifuging. After 5 minutes centrifuging, examination of the resulting cell layers showed that the lower part of the layers was white, due to the reduction of the methylene blue. It is well known that this reduction can only occur when oxygen is nearly absent,

even a trace of oxygen sufficing to maintain the redox potential of a biological system at too high a level for the reduction of the dye to occur. When a respiring layer of tumour cells containing methylene blue was viewed in a telemicroscope a thin blue layer was visible on the top next to the aerated Ringers solution or blood. The thickness of the blue layer varied with time, from 70 μ in a fresh preparation to over 200 μ after 3 hours (at room temperature). Unfortunately it is not possible to argue that the methylene blue test gives a perfect measure

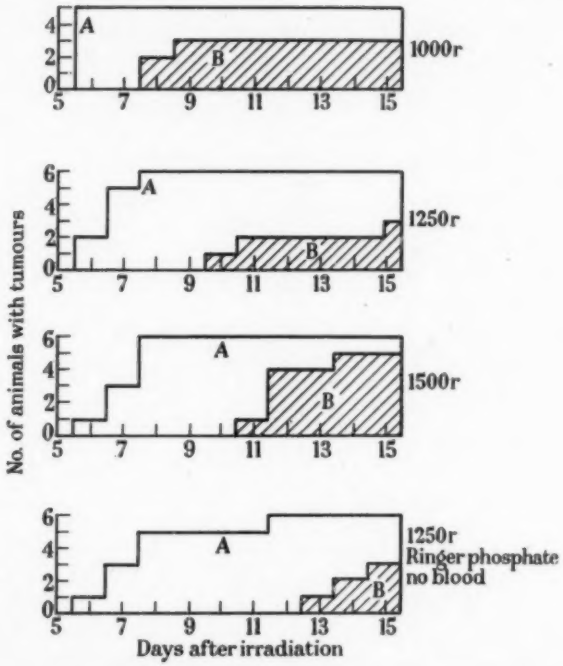

FIG. 3.—Growth of tumours from cells irradiated in arrangements A. and B. (Experiment of July 4, 1956.)

of the penetration of oxygen into a layer of cells, since the dye has complex effects on oxygen utilisation. Preliminary work by Dr. Dewey shows that the effect of dilute solutions of methylene blue on oxygen uptake of Ehrlich ascites cells is not great, and therefore that the dye does give a useful indication of the penetration of oxygen.

In all the " double layer " experiments, a 5-fold excess of H.R. cells was injected into each animal, along with the test cells. Further experiments were therefore carried out to test the effect of these H.R. cells on the growth of undamaged cells. Groups of 36 mice were injected with known numbers of tumour cells (counted in a haemocytometer) in a dilution series $1{\cdot}5 \times 10^6$, $1{\cdot}5 \times 10^5$ . . . 15 cells per mouse. Half of each group received, in addition, $7{\cdot}5 \times 10^6$ H.R. cells. The

time of appearance of visible ascites tumours was noted, and an estimate made of the time ($T_{\frac{1}{2}}$) when half of the animals which ultimately developed tumours had done so.

Over 80 per cent takes were recorded within the 7-week observation period provided the inoculum was greater than 1500 viable cells. When the inoculum was reduced below this level the percentage of takes fell and reached 50 per cent in the case of a 15-cell inoculum, whether or not heavily irradiated cells were added.

It will be seen that when numbers of intact cells were injected, the presence of an excess of H.R. cells reduced the time required for the appearance of tumours. This stimulating effect was not significant in the group receiving only 15 cells. The stimulating effect of H.R. cells was observed by Revesz (1955), using $1 \cdot 8 \times 10^6$

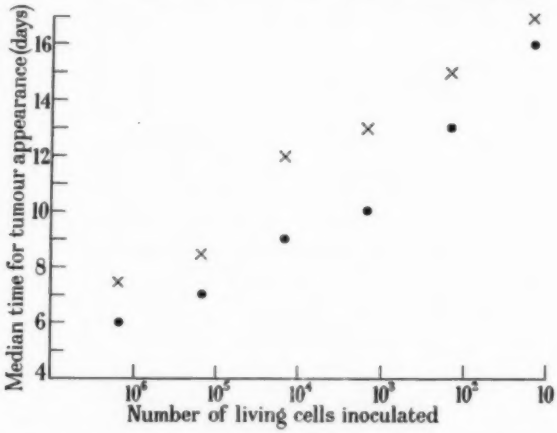

FIG. 4.—Time of appearance of tumours grown from inocula of unirradiated cells, with and without heavily irradiated cells.

× Undamaged cells only.
● Undamaged cells + $7 \cdot 5 \times 10^6$ H.R. cells.

unirradiated cells. He found, however, that when only 100 living cells were inoculated the presence of H.R. cells prevented the growth of tumours. He attributed this result to the development of tumour immunity, and it is perhaps not surprising that our results, with a different strain of mice, are not exactly the same in this respect.

It is clear that with our strain of mice and our tumour line the presence of a large excess of H.R. cells would not prevent tumour growth from a comparatively small number of untreated cells.

DISCUSSION

These results would seem to support the general picture of the radiosensitivity of metabolising layers, suggested by Hall, Hamilton and Brues (1952) and extended to certain histological types of human tumour by Thomlinson and Gray (1955).

In the model experiments all cells, irrespective of their situation when irradiated, had an equal opportunity to grow when inoculated into a new host.

In the human tumour, cells remote from stroma will depend upon the degeneration or phagocytosis of the more heavily damaged cells nearer to the stroma for renewal of their supply of nutrients. It may be that their growth is actually stimulated by the neighbouring heavily damaged cells but this has yet to be established. The undoubted stimulating effect which we have observed when heavily irradiated cells are inoculated along with viable cells into the peritoneal cavity entirely accords with a strong stimulating effect reported by Revesz (1956) in an extensive series of observations with solid as well as ascites tumours. The stimulating effect of H.R. cells has been made use of by T. T. Puck to obtain clonal growth from single mammalian cells, and two possible mechanisms for the effect have been suggested in a recent paper by Fisher and Puck (1956).

Our observations have a bearing upon the interpretation of many experiments in which the influence of oxygen and metabolic poisons on radiosensitivity has been studied. In plant tissues such as roots, microspores and seeds, in some situations within the living animal, and in all kinds of respiring tissues irradiated *in vitro*, such oxygen gradients as we have deliberately produced in our model experiments must exist, though their magnitude is generally unknown. In such situations, as well as through cell sedimentation in imperfectly stirred cell suspensions, the aerobic radiosensitivity will be correspondingly depressed. Reduced temperature or addition of respiratory poisons will diminish the gradients and increase radiosensitivity particularly the radiosensitivity of the deeper cells, as was observed in the case of tumour fragments irradiated aerobically in the presence of cyanide by Crabtree and Cramer (1934) and confirmed and interpreted by Hall, Hamilton and Brues (1952).

SUMMARY

By centrifugation of a suspension of ascites tumour cells models of certain histological types of human tumour, discussed in an earlier paper by Thomlinson and Gray (1955), have been set up whereby the behaviour and particularly the radiosensitivity of cells may be studied as a function of the availability of oxygen and other nutrients.

It has been found that tumour cells which are separated from their oxygen supply by a compact layer of metabolising cells no more than 250 microns thick become anoxic within a few minutes at room temperature, and are then much more resistant to radiation than the same cells when adjacent to their oxygen supply.

Ehrlich ascites tumour cells have been observed to respire normally for several hours at room temperature after exposure to 10,000 r, but they do not give rise to a tumour when inoculated into the peritoneal cavity. Compact layers of such cells form the metabolising barrier between the nutrients and tumour cells in the model referred to above. It was observed that when an inoculum of such heavily irradiated cells was injected with unirradiated cells into the peritoneal cavity the growth of the latter cells was markedly stimulated.

My thanks are due to Dr. L. H. Gray, for his help and advice, and to my colleagues Dr. Dewey and Dr. Deschner for their assistance.

We gratefully acknowledge the co-operation of the Mount Vernon Hospital Physics Department, who placed their radiation facilities at our disposal.

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IMMUNOLOGICAL DEMONSTRATION OF A SUBSTANCE IN RAT BLOOD ASSOCIATED WITH TISSUE GROWTH

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Received for publication December 14, 1956

IT was shown in an earlier paper (Darcy, 1955) that it is possible to distinguish the blood plasma of tumour-bearing rats from that of control rats by means of the gel-diffusion technique of Ouchterlony (1948). This technique is essentially the precipitin reaction carried out in a gel. A strong precipitating antiserum is prepared against the antigen, in this case rat plasma (a mixture of antigens), and the two reactants are allowed to diffuse towards one another through an agar gel. Precipitation of the antigen-antibody complexes occurs in the form of bands or lines, each of these representing one or more antigenic components of the original antigen mixture. Ouchterlony's method permits two such antigen mixtures to be compared in such a way that it is possible to tell at a glance whether there is a component in one which is absent from the other or whether a particular component is in substantially higher concentration in one than in the other.

The earlier study revealed several differences between the plasma of tumour-bearing rats and their controls, the most striking being : (1) The leading band of the normal plasma, i.e. the band that migrated in front of the others towards the antiserum, was considerably weaker in the cancer than in the normal blood. This band has since been found to be produced by serum albumin. (2) In the cancer plasma this albumin band was preceded by a group of faint lines (designated as " K " lines) which were not evident in the precipitin spectrum of the normal plasma. Their presence appears to be due partly to the receded position of the albumin band which thus " uncovers " them, and partly to higher concentration or higher speed of diffusion of the antigens they represent. (3) A line present in the cancer plasma spectrum which was absent from the normal. The substance which this line represents could be made to appear artificially in the plasma of normal rats by bandaging them tightly around the abdomen and not fasting them before bleeding. (4) Certain of the other antigens in the cancer plasma appeared to be in considerably lower concentration than in the normal, but some were higher.

The above characteristics were observed in the plasma of rats with well-established tumours. They were not seen in animals bearing the Walker tumour until about 6 days after its transplantation. A preliminary study (Darcy, unpublished) has since been made of human blood and has revealed exactly similar changes in the serum of patients with well-established tumours. But when serum from patients with early breast cancers was examined none of these differences from the normal were observed. Furthermore sera from some non-neoplastic diseases showed changes very like those of advanced cancer sera. Accordingly, in the present investigation attention was returned to rat blood in

an attempt to find changes during the very earliest stages of tumour growth. One striking change was found and is reported in this paper; it is shown however, to be characteristic of normal tissue growth as well as neoplastic.

METHODS AND MATERIALS

Antisera.—Two kinds of antisera were employed. For the first, 2 rabbits were injected with plasma from rats with large, actively growing, primary sarcomas induced with benzpyrene. The citrated plasma was emulsified in an equal volume of adjuvant consisting of 1·5 parts Arlacel A to 8·5 parts Bayol F. Four ml. of this emulsion was injected intramuscularly and this injection was repeated two weeks later. The animals were bled 11 days after the last injection. Ten months later a second antiserum was obtained from these rabbits after repeating the injection, this time subcutaneously. This last antiserum gave excellent discrimination between cancer and normal serum and was the one chiefly employed throughout the work. It was given the abbreviation A.K.P. (for anti-cancer plasma).

For the second type of antiserum an attempt was made to heighten any possible cancer characteristics of the immunizing rat plasma by first absorbing it with an antiserum prepared against normal rat plasma. To 1 volume of plasma obtained from a rat bearing a large transplanted sarcoma, were added 16 volumes of antiserum. After incubation for 2 hours at 37° C. and overnight at 6° C. the precipitate was centrifuged down and the supernatant used to immunize 2 rabbits in the manner described above except that inoculation was subcutaneous. A second antiserum was obtained from these rabbits some months later, after injecting an absorbed plasma obtained from a rat bearing a large hepatoma induced by feeding butter yellow. These antisera were given the abbreviation A.A.K.P. (for anti-absorbed cancer plasma.)

Animals.—All rats used were, unless otherwise stated, from a colony of albino animals originally derived from the Wistar strain. They had been bred by cousin matings. Males were used almost exclusively. They were bled from the heart under ether anaesthesia after overnight fasting. Rabbits were of various breeds obtained from dealers. They were bled from the marginal ear vein.

Gel diffusion technique.—Ouchterlony's method was followed in essentials. One per cent New Zealand agar, clarified and dissolved in 0·8 per cent NaCl buffered at approximately pH 7·3, was employed. Merthiolate at a concentration of 1 part in 5000 was incorporated in both the agar gel and in the antigens and antisera. The petri dishes in which the reactions were being carried out were kept in air-tight glass jars at room temperature. Two main types of configuration of the wells in the agar gel were used, one with three wells (cf. Fig. 1), the antiserum being placed in the bottom well and the two plasmas which were being compared, in the top wells; the other configuration was a 5-well modification of the first, with two additional antigen wells added to form a square (cf. Fig. 2). Plasmas for use in the gel plates were routinely diluted with 14 parts of citrate-saline (4 per cent sodium citrate, 1 part: 0·85 per cent NaCl, 4 parts), and sera with 19 parts of 0·85 per cent NaCl. Antisera were used either at full strength or diluted with one part of 0·85 per cent NaCl. No refilling of the wells was done.

Photographs were taken by ultraviolet light on blue-sensitive film. The first photograph of each plate was usually taken at 2 to 5 days,after the start of the experiment, a second at 9 to 11 days, and a third at 2 to 3 months.

RESULTS

Discriminatory Power of the Antisera

The first object of the investigation was to see whether the pattern of precipitation shown by the blood serum from an animal with an early cancer differed in a characteristic way from that of normal rat serum. In practice, this amounted to a search for any asymmetry in the total precipitate pattern when plasma from rats bearing a 5-day-old Walker tumour implant was compared side by side with normal plasma. The first indication that any such difference existed was obtained when an antiserum against the absorbed cancer plasma (A.A.K.P.) was used. Fig. 1 shows the result. This antiserum gave a weak and simple spectrum of only 4 lines as opposed to the usual 20 or more. The absorption of the antigen used to prepare this antiserum had greatly reduced the number of its component antigens. The figure shows no lines present on the cancer side which are not also present on the normal side. The only apparent difference is that one of the 4 lines (the second, counting from the antiserum well) is broader on the cancer side and extends nearer to the antiserum well. This indicates that the substance responsible for this line is in higher concentration in the cancer blood than in the normal. This not very striking difference was subsequently confirmed using other 5-day Walker tumour plasmas and fresh normal plasmas.

When the antisera prepared against the untreated rat cancer plasma (A.K.P.) were tested, one of them, too, gave a discrimination between 5-day Walker tumour plasmas and control plasmas. The essential difference was again a line in the spectrum which was stronger on the cancer side than on the control side (Fig. 2). In this case the difference was more striking. Other, less striking, differences were also noted and will be indicated later. It was clear therefore, that both types of antisera showed that there was a substance present in normal rat blood which was in noticeably higher concentration in the cancer blood. The magnitude of this concentration difference was found to be considerable (see below). The question next arose whether the substance was the same with either antiserum. This could be tested by comparing the two antisera in a 3-well plate, with the antigen (Walker tumour plasma) in the bottom well. The result was a clear-cut " reaction of identity " or arc-formation between the two lines in question, indicating that the antigen that produced them was one and the same. For most subsequent experiments the second antiserum (A.K.P.) was used because it showed the above concentration difference more strikingly (due to its higher content of the corresponding antibody) and could discriminate smaller concentration differences, and because it showed concentration differences in other antigens.

Walker tumour 1 to 5 days old

The next step was to find how early after implantation of the Walker tumour the above change could be detected in the animal's blood. Ten rats were implanted subcutaneously with pieces of healthy Walker tumour by trocar. Twenty-four hours later 2 of them were bled from the heart ; another pair were bled at 2 days, other pairs at 3, 4, and 5 days. The tumour grafts were examined at autopsy.

The plasmas of all these rats showed a strong increase in the antigen, as had the 5-day tumour plasmas. Results with the A.K.P. serum indicated that the

increase was greater at 3 days than at 1 day, and later results showed that the concentration of the antigen in the serum of a rat bearing the tumour for 13 days was about twice as great as at 5 days. One of the 5-day plasmas was comparatively weak in the antigen, and autopsy notes stated that the tumour in this animal was much smaller and in poorer condition than in the other 5-day animals. This suggested that the graft in question may have been starting to regress as happens in a proportion of these Walker tumour grafts.

To confirm the effect at 24 hours, 10 more rats bearing the tumour for that period were bled. All plasmas showed the strong increase in the antigen concerned (Fig. 3). Other differences noted were (*a*) the line just ahead (i.e. nearer the antibody well) of the one just described was also stronger in the cancer blood than in the control, and (*b*) the first line of the spectrum (produced by albumin) was somewhat weaker in the cancer blood.

Other Cancer Bloods

Yoshida sarcoma

To see whether the same change occurred with other tumours and other rat strains, the Yoshida sarcoma was implanted subcutaneously in 8 rats of the BD-1 strain in which it grows almost as rapidly as the Walker tumour does in the albino stock rat. Animals were bled in pairs at 1, 2, 4, and 6 days after transplantation of the tumour. Two normal BD-1 rats of the same batch provided control plasmas. The gel diffusion test showed that the 1-day plasmas were very little different from the controls, although one was slightly stronger in the antigen than the controls. The 2, 4, and 6-day plasmas however, showed a strong increase in the substance exactly as had occurred with the Walker tumour (Fig. 4).

August tumour

This tumour which arose in the inbred August strain rats as a mammary carcinoma grows extremely slowly in its strain of origin. It takes almost 2 weeks after transplantation to reach a measurable size and about 3 months to kill its host (compared with about 3 weeks for the Walker tumour). Ten August strain rats were implanted with the tumour using rather larger grafts than usual in order to favour any possible role played by graft-necrosis in the antigen increase. The rats were bled in pairs, at 1, 2, 4, 8, and 16 days. One normal rat of the same batch provided control blood. Of the rats bled up to 8 days only one of the 2-day ones showed a concentration of the antigen which was definitely stronger than in the control blood ; it had been noted at autopsy that this animal had a larger and better attached tumour than its companion. Several of the others showed a slightly stronger concentration of the antigen than the control, but without a quantitative method they could not be regarded as significant. Only the two 16-day plasmas showed a strong increase of the antigen ; by this time the tumours had reached a fair size, about 3 cm. $\times$ 2 cm. measured through the skin (Fig. 5).

These results suggested that the increase of the antigen in the blood was in some way associated with tumour size or tumour growth.

Benzpyrene-induced sarcomas

This experiment was performed to see whether the antigen increase occurred with non-transplanted tumours, and if so, how early in the process of tumour

induction it would appear. Stock albino rats were implanted subcutaneously with small pellets of 3 : 4-benzpyrene which normally gives rise to a sarcoma in about 75 per cent of the rats. The first tumours appear at about 5½ months after the implantation. Six of these rats were bled at 1½ months when there was no trace of palpable tumour. All but one were negative in the gel test (i.e. showed no increase in the antigen). Five more rats were bled after 4½ months. None had palpable tumours. All were negative in the gel test except one. This one was the only female present. Six more of these rats were bled at 5½ months after implantation of the pellet. One of them had a small visible lump and another had a barely palpable lump around the pellet. The remaining rats showed no evidence of a tumour at this time. When the plasmas were tested, those from the 2 rats with palpable lumps, and plasmas from two of the other rats, showed definitely higher concentrations of the antigen than did the controls. But none showed very large increases.

Control Plasmas

The first experiments were carried out comparing the cancer plasma with normal rat plasma. The normal was invariably negative, i.e., never showed a concentration of the antigen in any way comparable to that in the blood of animals with the Walker tumour. Various other types of non-cancerous control plasmas were then taken to see if the phenomenon was cancer specific.

Bandaged and fed controls

The previous investigation had shown that when rats were bandaged around the thorax and abdomen, and were not fasted before bleeding, a new antigen appeared in their plasma. It was important, therefore, to see whether this procedure affected the antigen under consideration here. Ten rats were bandaged fairly tightly around the lower thorax and upper abdomen with gauze bandage, and over it plaster bandage about 2 inches wide. Another 10 were similarly bandaged but more in the abdominal region. Two rats from each batch were bled daily for 5 days. None were fasted before bleeding. Only one of the 20 plasmas showed a concentration in the antigen which could be regarded as definitely higher than the control. At the same time it was noted that, in animals bearing the Walker tumour, fasting before bleeding did not affect the antigen increase.

Transplants of normal tissue

To obtain control animals comparable with the tumour-bearing ones, a series of grafting experiments were performed in which various normal tissues were grafted between rats of the albino stock. In the first experiment 10 rats were grafted subcutaneously by trocar with pieces of kidney from another rat. Two rats were bled out each day for 5 days thereafter. One of the 5-day specimens was " positive ", i.e. it showed a concentration of the antigen as strong as one of the weaker Walker-tumour plasmas. The rest were negative or else showed increases which could not be declared significant by the present method.

Another 10 rats were similarly grafted with embryonic tissue, a whole 8- or 9-day rat embryo being implanted subcutaneously in each host by trocar. All the plasmas were negative in the gel test. The experiment was then repeated using 19-to 20-day rat embryo liver in a volume comparable to the Walker tumour

implants. One of the hosts bled at 4 days gave a clear-cut positive (Fig. 6); it had been noted at autopsy to have a much larger graft than its companion rat. One of the animals bled at 24 hours showed a concentration of the antigen which was intermediate between the normal and the Walker tumour level. The rest were negative.

Wound healing and tissue regeneration

The last results made it unlikely that the phenomenon of the antigen increase was specific for tumour growth and suggested that it was associated with active growth of any tissue. To test this hypothesis, regeneration experiments were set up.

Ten rats had the left lobe of the liver removed and were bled in pairs at 24 hours, 2, 4, 8, and 16 days. This was designed to cover the main period of regeneration. The mass of the liver is known to be restored by 14 days after partial hepatectomy and the peak of mitotic activity occurs at between 2 to 8 days. Mitotic activity has begun by 24 hours. The results were as follows: All the hepatectomy plasmas except the 16-day ones were positive; furthermore, they were strongly positive, except the 1-day specimens and one of the 8-day ones which were relatively weak but nevertheless showed considerably stronger concentrations of the antigen than the normal (Fig. 7). The 16-day plasmas were negative.

The experiment was repeated for kidney regeneration. Ten rats had one kidney removed and were bled as above. The 1-day specimens were positive, but weakly so. Of the 2-day specimens, one was weakly positive the other strongly. Both the 4-day specimens were moderately strong positives. The 8-day specimens were negative although one had a somewhat higher concentration of the antigen than the controls. The 16-day specimens were negative.

Finally, the experiment was repeated for skin regeneration. Ten rats were taken as before and a circular area of skin on their backs, 2·5 cm. in diameter, was excised in full thickness under sterile conditions. No sutures were applied but sterile *tulle gras*, gauze bandage, and finally the protective plaster bandage. The plasmas were tested as usual and gave the following results: 1-day plasmas, negative; 2-day plasmas, one negative and one doubtful; 4-day plasmas, positive but not strongly so; 8-day plasmas, negative; 16-day plasmas, one negative and one doubtful.

As a result of this finding sham operations were performed as if for hepatectomy. This procedure caused a definite but not large increase in the concentration of the antigen. The maximum concentration reached was not as great as that for the dermatectomy experiment. Nevertheless it suggested that a considerable part of the antigen increase in the nephrectomy experiment (where the increase was only moderate) was due merely to the laparotomy.

Pregnancy plasmas

As a further test for the hypothesis that the antigen increase was associated with actively-growing tissue, plasmas were tested from rats with pregnancies ranging from 10 to 20 days duration. All showed a very strong increase in the antigen—at least as strong as in the tumour blood. The weakest of the series was from the rat with the earliest pregnancy, namely 10 days (Fig. 8).

Immature versus mature rat blood

If the tissue growth hypothesis is true, it would be expected that the blood of young and rapidly-growing rats would have a higher concentration of the substance than that of adult rats. To test this, sera from rats, 1, 2, 4 and 8 weeks of age were compared in one gel plate. Four separate experiments were made. Each one showed that the blood of a 1-week-old rat had a distinctly higher concentration of the substance than that of an 8-week-old animal.

Quantitative Aspects

It was possible to obtain a rough estimate of the increase in concentration of the substance under study by two methods. The first was to take a normal plasma and find how high a concentration of it had to be employed in order to give a precipitate line identical with that in a cancer plasma used at the standard 1 : 14 dilution. The end-point was taken where the two lines were as nearly as possible equal in density, width, and distance from their own antigen wells. When a normal plasma was compared with a 1-day Walker tumour plasma in this way, it was found that the normal plasma had to be used in a 5 times higher concentration than the cancer plasma. The other method is one under development in this laboratory. In it, an extraneous " marker " line is introduced into the spectrum at a fixed point between the wells. The position of the line under study is noted in relation to the marker line and compared with its position in a series of standard dilutions. This method yielded the same result as the first, and indicates that the increase in concentration of the antigen under consideration is about five-fold at 24 hours after implantation of the Walker tumour. In another experiment, a 13-day Walker tumour serum was examined and found to contain approximately 10 times as much of the substance as a normal control.

It could also be observed that the concentration of the antigen which was arbitrarily chosen as being " positive " (implying a significant difference from the normal) was certainly not less than 100 per cent greater than that in any of the normal plasmas studied.

Characterization of the Antigen

The first clue as to the nature of the substance was obtained when tests were done on a sample of rat serum albumin known to contain 4 per cent of α-globulin. This sample was prepared by means of ether fractionation and kindly supplied by Dr. Margaret Mackay. When tested in the Ouchterlony plates it gave several subsidiary bands of precipitate in addition to the main albumin one. It was found that one of the subsidiary antigens gave a reaction of identity with the antigen under study here. It was presumed therefore that the substance was probably an α-globulin. Confirmatory evidence was obtained by means of the technique of immune-electrophoresis of Grabar and Williams (1953). Here it was found that the substance appeared to migrate with the α-globulins, although the possibility that it might be a fast-moving β-globulin could not be entirely ruled out.

A chemical approach was then made to the problem, and at the suggestion of Professor E. Boyland, rat cancer serum was precipitated with sulphosalicylic acid, and the supernatant, after suitable dialysis, was tested in the gel plates. It was found to give three bands, apart from traces of other antigens. One of the bands gave a reaction of identity with the antigen under study (Fig. 9). It can therefore be presumed to be a mucoprotein.

Site of Origin of the Substance

Preliminary studies have yielded no clue as to the site of origin of the substance. A saline extract made of non-necrotic, washed, and homogenized Walker tumour tissue contained no more of the substance than would be expected from the small amount of blood contamination still remaining. The same result was obtained with an extract of well-washed liver cells of a pregnant female rat.

DISCUSSION

The present experiments were begun largely in the hope of finding some characteristic early change in the blood of cancerous rats which might be of diagnostic value. No antigenic substance was found in the plasma of rats bearing early tumours which was not also present in normal rat plasma. However, one striking quantitative difference is reported, namely, an antigenic substance in normal blood which undergoes a large increase in concentration during tumour growth. Twenty-four hours after implantation of the Walker tumour the increase in concentration of this substance is approximately five-fold. When the slow-growing August tumour was employed no considerable increase took place until between 8 to 16 days after transplantation. During benzpyrene carcinogenesis the substance had already increased in the blood when the earliest tumours were present. Further experimentation showed that the phenomenon occurred in non-cancerous rats which possessed actively-growing tissue, e.g. in animals regenerating liver or kidney or skin and particularly in pregnant females. The substance was also in distinctly higher concentration in the blood of 1-week-old rats than in that of 8-week-old rats.

EXPLANATION OF PLATES

FIG. 1.—Petri dish showing antigen-antibody reaction in agar gel. Rat cancer plasma (K) and normal plasma (N) have been allowed to diffuse from the upper wells to meet the anti-serum (A.A.K.P.) diffusing from the lower well. The only asymmetry is in the width of the middle band of the precipitate. The cancer plasma is from a rat bearing a 1-day-old Walker tumour graft.

FIG. 2.—The cancer plasma (K) is from rats bearing 3-day-old Walker tumour grafts. The antiserum (A.K.P.) was prepared against cancer plasma. The two cancer plasmas give a strong band of precipitate which is almost absent from the normal rat plasma (N).

FIG. 3.—1-day Walker tumour rat plasma (K) contrasted with normal rat plasma (N). The cancer plasmas show a strong band in mid-spectrum which in the normal is diffuse. The line ahead of this is also stronger in the cancer blood, and there are other differences.

FIG. 4.—4-day Yoshida sarcomar at plasma (K) contrasted with normal rat plasma (N) to show the stronger mid-line of the cancer plasma.

FIG. 5.— 16-day August tumour plasma (K) showing a much stronger middle band than the normal (N). The antiserum (A.K.P.) was here used in a dilution of 1 : 1.

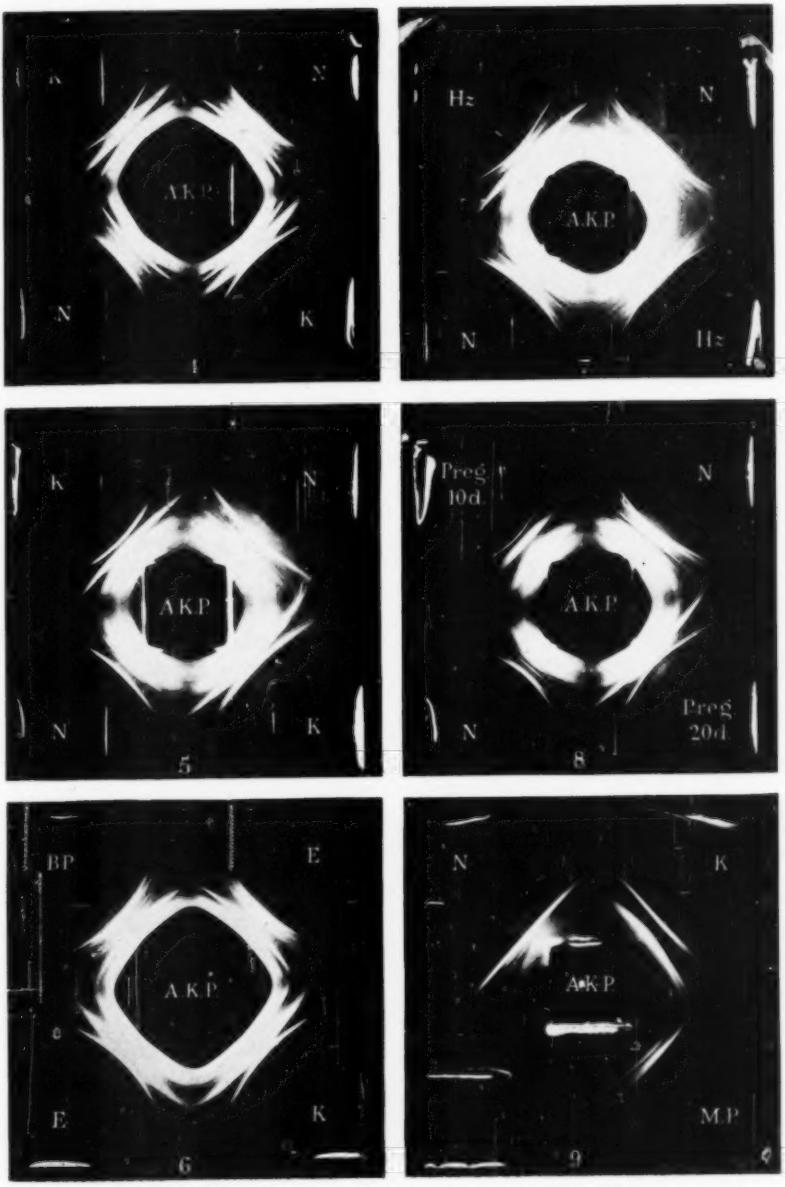
FIG. 6.—Plasma from rats bearing embryonic liver grafts for 4 days (E) contrasted with a 4-day Walker tumour plasma (K) and a control plasma from a rat with benzpyrene implanted (BP) which was negative. The bottom (E) plasma is almost as strong in the antigen as the Walker plasma.

FIG. 7.—Plasma from rats 8 days after partial hepatectomy (Hz) is contrasted with normal rat plasma (N). The mid-band is much stronger than the normal in one of the hepatectomy plasmas, but only slightly stronger in the other.

FIG. 8.—Plasma from rats pregnant for 10 and 20 days is contrasted with the normal.

FIG. 9.—Normal rat serum (N) and 13-day Walker tumour serum (K) are placed in the top wells to show the position of the antigen line under study. It is much stronger in the K serum. The third well contains a mucoprotein preparation of 13-day Walker tumour serum (M.P.). It gives 3 main lines, at least one of which is seen to arc with the strong antigen of the K serum.

K
N
A.K.P
1
K
N
A.K.P
N
K
2
K
N
A.K.P
N
K
3


K
N
A.K.P.
N
K
1
Hz
N
A.K.P.
N
Hz
K
N
A.K.P.
N
K
5
Preg.
10d.
N
A.K.P.
N
Preg.
20d.
8
BP
E
A.K.P.
E
K
6
N
K
A.K.P.
M.P.
9

It seems very likely therefore, that the increase in this substance is associated with active tissue growth rather than with a phenomenon such as tissue necrosis. For the necrosis hypothesis does not explain the negative results obtained with the majority of normal tissue grafts which certainly become necrotic. Nor could necrosis account for the positive results with pregnancy, regeneration, and 1-week-old rat plasma. On the other hand the increase of the substance appears to be related to the growth rate of the various tumours and to the extent and duration of the growth of normal tissue. The results suggest that the fundamental correlation might be with the overall mitotic rate of the body. The number of mitoses in a Walker tumour at 24 hours after transplantation could well be as great as in a much larger and later August tumour, or in a regenerating liver lobe. The failure to obtain a significant increase of the substance with most normal tissue grafts could be explained by their relatively low growth rates and the small amount of tissue involved. The level of the substance in normal blood could be accounted for by the mitotic activity needed for normal growth, maintenance, and repair. In short, the hypothesis that the concentration of the substance in the blood is directly associated with the total mitotic activity of the body seems worth considering.

It is more probable, however, that the fundamental correlation is with a secondary effect of tissue growth. Since the substance is apparently a mucoprotein, it may be merely an expression of connective tissue activity. Connective tissue activity cannot be ruled out in any of the experimental situations described. In fact it is difficult to rule it out for any tissue growth *in vivo*. It is worth observing, however, that the high level of the substance in the blood during liver regeneration appears to be out of proportion to the visible fibroblast activity. The actual laparotomy can only account for a minor part of the increase.

The fact that the substance appears to be an α-globulin and a mucoprotein raises the question of its identity or otherwise with similar substances reported by other authors. Numerous reports are to be found in the literature of increases of α-globulins and mucoproteins in cancer and other conditions. Butler (1951) working in this laboratory on Brdička's (1939) polarographic serum test found that this method of testing human serum, although useless as a diagnostic tool for cancer because of the high proportion of false positions it gave with other diseases, was nevertheless useful for predicting the course of the disease. A serum mucoprotein appeared to be responsible for the polarographic behaviour (Winzler and Smyth, 1948), and this protein appeared to migrate with the α-globulins (Boyland, Butler and Conway, 1951). It appears likely therefore that the substance dealt with in the present paper may be the analogous substance in the rat to that dealt with in man by the polarographic method. However, since sulphosalicylic acid deproteinization of rat serum left at least three antigens, only one of which undergoes an increase during tissue growth and is the subject of the present paper, it seems that the gel diffusion method is more highly selective and could give more accurate results.

Shetlar (1952) states that the polysaccharide content of the albumin fraction of serum appears to be elevated in all cases in which tissue proliferation occurs but that the polysaccharide content of the α-globulin fraction increases in fever. This is not in accord with the present results.

Hokkanen, Pyörälä and Taipali (1956) studying the serum mucoprotein of rats bearing on ascites tumour found that it reached its maximum after the

tumour began to regress. This argues against the cell-division hypothesis proposed earlier, but it may be remarked (*a*) that besides the present antigen there are other mucoprotein components in rat serum which might be responsible for the high level and (*b*) it cannot be ruled out at once that there is less overall cell-division going on in the body after the tumour begins to regress—the activity of normal tissues, especially lymphoid and myeloid tissue, has to be taken into account.

Lockey, Anderson and Maclagan (1956) in a survey of the mucoprotein content of serum and urine report that high levels of these substances are frequently found in inflammation and in the collagen diseases as well as in cancer. Furthermore, surgical operations almost always produced a marked rise. In the light of the present results it is interesting to consider whether this rise in human serum mucoprotein might be due to one mucoprotein only, so that if this substance alone could be followed greater precision would result.

A recent report which is of great interest in the light of present findings is that of Nisselbaum and Bernfeld (1956). These authors isolated the α-globulin glycoproteins of the plasma of normal mice and tumour-bearing mice. The substances were studied chemically and physically and found to be distinct from one another. The protein preparation from the tumour-bearing animals behaved on electrophoresis like a nearly uniform substance over a wide range of pH, while that from normal mice was a mixture of several proteins. The authors conclude, " it appears likely that the α-globulin isolated from tumour-bearing mice is one of the regular constituents of normal mouse plasma α-globulin, the concentration of which increases during tumour growth ". The present findings in the rat are in harmony with this statement. On the other hand the earlier suggestion of Bernfeld and Homberger (1955) based on negative results obtained with normal and embryonic tissue grafts, that the increase of the plasma α-globulin is specifically associated with neoplastic growth, is opposed by the present results. It was found that if the growth of normal tissue was sufficiently vigorous, then positive tests were obtained.

Finally, there are one or two questions concerning the gel diffusion results that require discussion. The first is about certain assumptions made in interpretation of the precipitin lines. The assumption was made throughout that since the antigen under study gave a single line it was therefore a single substance. This need not necessarily be so. But since a wide range of antigen dilutions failed to split the line into components, the singleness of the antigen must be assumed until there is good evidence to the contrary.

The other assumption was that if a line is found to be nearer the antiserum well than another line with which it gives a " reaction of identity ", that the antigen responsible is in higher concentration in the first case. This is usually a safe assumption. However, if the first antigen should be of lower molecular weight (e.g. because it was partly hydrolysed) and therefore diffused faster than the other, the position-difference might be obtained without any actual increase of antigen. The author has had exactly this experience with the fibrinogen line of rat plasma which had become infected. However, in this eventuality the faster-diffusing antigen would be expected to give a line of different density and appearance, if not a reaction of " partial identity ". Since there was no evidence for this, the more straightforward interpretation was assumed here.

There were other antigenic changes noted in early tumour blood and it is hoped to present a fuller study of these at a later date.

SUMMARY

An attempt has been made to discriminate the blood plasma of rats bearing very early tumours from that of control rats. The method used was the analysis of plasma antigens by means of the gel-diffusion technique of Ouchterlony (1948).

No antigenic substance was found in the plasma of rats bearing early tumours which was not also present in the control plasma. However, one striking quantitative difference is reported ; namely an antigenic substance in normal blood which undergoes a large increase in concentration during tumour growth. Twenty-four hours after implantation of the Walker tumour this increase is approximately five-fold. When the slow-growing August tumour was employed no substantial increase was observed until after 8 to 16 days following transplantation. During benzpyrene carcinogenesis the substance had already increased in the blood when the earliest tumours were present.

Further experiments showed that the phenomenon occurred in non-cancerous rats which possessed much actively-growing tissue, e.g. in animals regenerating liver, kidney or skin, and also in pregnant females. The substance was found in distinctly higher concentration in the blood of 1-week-old rats than in that of 8-week-old rats.

The substance appears to be a mucoprotein which migrates electrophoretically with the α-globulins. Its relation to similar substances reported on the literature is discussed.

The working hypothesis is suggested that the substance is directly associated with cell-division.

The author is much indebted to Dr. B. Cinader who carried out the immune electrophoresis, to Professor E. Boyland for advice on biochemical procedure and to Miss Teresa Scruton for outstanding technical assistance.

This investigation was supported by grants to the Royal Cancer Hospital and Chester Beatty Research Institute from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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STUDIES ON ISOLATED TUMOUR MITOCHONDRIA. PHOSPHATE RELEASE FROM ADENOSINE DI- AND TRIPHOSPHATES

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Received for publication January 11, 1957

PREVIOUS work has shown that mitochondria prepared from certain tumour strains in isotonic sucrose containing 0·001 M EDTA,* exhibited higher ATP- and DPN-splitting activities than the mitochondria from other tumours (Emmelot, Bos and Brombacher, 1956). When the former were added to fresh liver mitochondria, the octanoate- or BHB-oxidation of the liver mitochondria became completely inhibited in the combined system (Emmelot and Bos, 1955*a*, 1955*b*, 1956*a*). It was found that the DPNase inhibitor NAA, or DPN itself, re-established the oxidation when the D-isomer of BHB served as a substrate in the combined system of liver and tumour mitochondria (Emmelot and Bos, 1955*b*, 1956*a*). The oxidation of the L-isomer of BHB, however, still remained blocked under the latter conditions. This difference in behaviour of the liver mitochondria in the presence of the tumour mitochondria in respect to the oxidation of the two isomers was explained on account of the co-factors necessary for oxidation, which have been shown to be dissimilar (Lehninger and Greville, 1953). The latter authors found that the oxidation of D-BHB by rat liver mitochondria proceeded independently of ATP in that the free acid was oxidized, whereas L-BHB needed ATP for the formation of its CoA-derivative, L-BHByl-CoA being the form in which the L-isomer was oxidized. DPN acted as coenzyme of both the D-BHB and the L-BHByl-CoA dehydrogenases.

Thus, by adding DPN to the combined system of liver and tumour mitochondria, the oxidation of D-BHB might easily be repaired, but the "ATPase" activities of the tumour mitochondria would render the oxidation of L-BHB impossible by depriving the liver mitochondria of ATP.

In the present communication direct evidence is presented for the uncoupling action of certain tumour mitochondria on the phosphorylative processes of the liver mitochondria. Related experiments dealing with the phosphate release from ATP and ADP by these and other tumour mitochondria under various experimental conditions are also described and compared with the results obtained with mouse liver mitochondria.

MATERIALS AND METHODS

Tissues.—Livers were collected from mice of several inbred strains and their F_1 hybrids. Unless otherwise stated transplanted mouse tumours which have been described previously (Emmelot and Bos, 1955*a*) were used.

* Abbreviations are used as follows: EDTA = ethylendiamine tetracetate; AMP, ADP, ATP = adenosine mono-, di- and triphosphates; "ATPase" = adenosine triphosphatase; DPN = disphosphopyridine nucleotide; DPNase and TPNase = di- and triphosphopyridine nucleosidase; NAA = nicotinamide; BHB = β-hydroxybutyrate; CoA = Coenzyme A; P_i = inorganic orthophosphate; P : O = ratio of micro-moles of inorganic phosphate esterified to the micro-atoms of oxygen utilized; 2 : 4-DNP = 2 : 4-dinitrophenol.

Preparation of mitochondria.—Liver mitochondria were isolated in 0·25 M sucrose and tumour mitochondria in 0·25 M sucrose containing 0·001 M EDTA pH 7·4. The mitochondrial pellets, which were spun down by centrifugation at 0° C. during 10 minutes at 5000 × g after removal of nuclei and cell débris, were always washed twice by resuspension and centrifugation at 18,000 × g. Special care was taken to remove the fluffy layer as completely as possible.

Preparation of mitochondrial sub-fractions.—Mitochondria from 1 g. of tissue were suspended in 1 ml. of distilled water during 60 minutes at room temperature. The swollen mitochondria were then disrupted by homogenization for 1 minute, with pyrex glass powder in a tight-fitting homogenizer of the Potter-Elvejhem type held in cracked ice. Centrifugation for 5 minutes at 700 × g was carried out to remove the glass powder, followed by 30 min. at 20,000 × g. The latter procedure yielded a pellet and a supernatant. The supernatant was clear yellowish in the case of liver mitochondria but (faintly) turbid in the case of tumour mitochondria. The pellet was washed once with 2 ml. of distilled water. Pellet and combined supernatants were then made up with distilled water in the relation 1 : 2 respectively to the smallest volume possible. 0·1 ml. (pellet) and 0·2 ml. (supernatant) aliquots were added to the Tris-KCl-$MgCl_2$ medium of Chappell and Perry (1954) which contained either ADP (0·005 M) or ATP (0·005 M). Final pH 7·4. Incubation was carried out at 27° C. for 20 minutes.

In a number of experiments, after removal of the glass powder, the suspension of the disrupted mitochondria was brought to pH 5·0 with 1 per cent of acetic acid and centrifuged. The resulting sub-fractions were neutralized with dilute KOH.

It was not investigated whether under the conditions chosen for the disruption of the mitochondria maximal recoveries of the activities were obtained. In fact it is suspected that part of the enzymes are inactivated due to the local heating by friction during the energetic grinding of the particles.

Determinations.—Inorganic phosphate release from ADP and ATP by whole mitochondria was measured under the same conditions as those described for the sub-fractions. Care was taken to use approximately similar quntities of mitochondria (based on mg. nitrogen) in the experiments since the ATPase activites have been shown not to be proportional to enzyme concentration. The specific activities of the ATPase were smaller the higher the enzyme concentration used.

Phosphate was determined by the method of Fiske and Subbarow (1929). The conventional Warburg technique was used to measure oxygen uptake during oxidation of DL-hydroxybutyrate or pyruvate at 27° C. Acetoacetate was determined according to Thin and Robertson (1952). ATP and ADP were products from Zellstoffabrik Waldhof (Wiesbaden-Germany); the compounds were 75 and 80 per cent pure as assayed enzymatically with hexokinase plus glucose-6-phosphate dehydrogenase (mouse liver) in the absence and presence of myokinase (rabbit muscle), respectively. DPN (95 per cent pure) was obtained from Boehringer u.S. (Mannheim, Germany) and freshly prepared human serum γ-globulin and albumin from the Central Laboratory of the Netherlands Red Cross (Amsterdam). Another albumin preparation obtained from the same Laboratory five years ago had been kept at room temperature by us; it was slightly yellow coloured and is designated in the text as aged albumin. Neutral solutions of the proteins were added in a final concentration of 2 per cent.

The experiments illustrated in the tables and figures are representative examples from several test series.

RESULTS AND DISCUSSION

Oxidative phosphorylation in a combined liver-tumour mitochondrial system

If DPN addition re-establishes the oxidation of the D-isomer but not that of the L-isomer of BHB in the combined liver-tumour mitochondrial system incubated in the presence of DL-BHB, only 50 per cent of the theoretical oxygen consumption necessary for the conversion of DL-BHB to acetoacetate may be expected. This was actually found. Acetoacetate production was likewise approximately half of that shown by the liver mitochondria incubated separately. Table I illustrates one such experment in which the mitochondria from the adrenal cortex carcinoma T17572 were used. It should be noted that the tumour mitochondria *per se* were not able to oxidize DL-BHB under the conditions used in these experiments.

TABLE I.—*Oxidation of* DL-*β-hydroxybutyrate by Liver Mitochondria Incubated Separately and Together with Tumour Mitochondria*

0·3 ml. of a liver mitochondrial suspension in 0·25 M sucrose was added to Warburg flasks containing 100 μ-moles KCl, 21 μ-moles K-phosphate buffer pH 7·4, 10 μ-moles ATP, 0·01 μ-moles cytochrome C and 35 μ-moles $MgSO_4$.
0·3 ml. of a tumour mitochondrial suspension in 0·25 M sucrose containing 0·001 M EDTA was added to two of these flasks. In one of the latter flasks in addition 1·6 μ-moles DPN were present. After temperature equilibration 12 μ-moles DL-BHB were tipped in. Total volume 1·6 ml. The centre well contained 0·1 ml. 3NKOH. Incubated 60 minutes at 27° C.

Mitochondria from	N present per flask (mg.)	Oxygen uptake (μ-atoms)	Acetoacetate formation (μ-moles)
Liver	1·55	12·0	10·0
Liver+T17572	1·55+1·24	0·2	0·3
Liver + T17572 + DPN	1·55+1·24	6·6	5·2

That the ATP-dependent oxidation of the L-isomer of DL-BHB remained blocked was proven by experiments in which the isomers of BHB were incubated separately with the combined liver-tumour mitochondrial suspensions (Emmelot and Bos, 1956*a*). The nature of this inhibition has now clearly been demonstrated by measuring the phosphate uptake during the re-established oxidation of the D-isomer in a DPN-fortified liver-tumour mitochondrial system incubated with DL-BHB. As illustrated in Column 4 of Table II no net uptake of P_i occurred during this oxidation. The P : O ration of 0·1 which was found illustrated that the tumour mitochondria acted as uncouplers of the phosphorylations association with the oxidation of D-BHB by the liver mitochondria. Hence no ATP became available for the required conversion of L-BHB to L-BHByl-CoA and, in consequence, the oxidation of this isomer remained depressed.

Similar results were obtained with the mitochondria from other tumours, like the sarcoma UV256, the sarcomatoid ovarian tumour T24202 and primary azodye-induced rat hepatomas and transplants thereof.

In the present experiments oxygen and phosphate uptakes were measured after 15 minutes of incubation. During this period both the oxidation of BHB

TABLE II.—*Oxidative Phosphorylation of Liver Mitochondria Incubated Separately and Together with Tumour Mitochondria*

Experimental conditions as in Table I. Instead of ATP 10 μ-moles ADP were present. The μ-moles P_i were lowered to 14·4. From the side arm of the flasks 12 μ-moles DL-BHB, 40 μ-moles glucose and 30 Kunitz-McDonald units hexokinase were added. Incubated for 15 minutes at 27° C.

Mitochondria from	N present per flask (mg.)	DL-BHB	P_i found (μ-moles)	$-\Delta P_i$ (μ-moles)	$-\Delta O$ (μ-atoms)	P : O
Liver	1·46	Absent	13·5	3·9	3·0	1·3
		Present	9·6			
Liver + T17572 + DPN	1·46+1·06	Absent	18·1	0·2	2·1	0·1
		Present	17·9			

by the liver mitochondria and that by liver plus tumour mitochondria with DPN present, is incomplete. The liver mitochondria showed P : O ratios in the range of 1·2–1·5 (in the experiment illustrated in Table II a ratio of 1·3 was found). The latter values may be higher when fluoride is added. In the present type of experiments fluoride had to be absent for obvious reasons. In the earlier experiments (Emmelot and Bos, 1955*b*, 1956*a*; compare Table I) ATP was added instead of ADP and under this condition it was found that the oxidation of DL-BHB or D-BHB by the liver-tumour mitochondrial system fortified with DPN, proceeded initially at a higher rate than the oxidation of the liver mitochondrial controls. This could have been due to the "ATPase" activities of the tumour mitochondria since it is known that a "high energy" phosphate trapping system enhances mitochondrial oxidations. Now that ADP was used as phosphate acceptor in the present experiments the oxidation in the liver-tumour mitochondrial system was equal to or somewhat less than that shown by the liver mitochondria incubated separately. This observation might give some weight to the above assumption.

In further experiments it was found that fluoride (0·01 M) partly relieved the inhibition exerted by the tumour-mitochondria on the liver mitochondrial oxidation of DL-BHB *in the absence* of DPN (Emmelot and Bos, 1956*b*). When the two isomers were studied separately it appeared that fluoride addition repaired the oxidation of the D-isomer, as shown in Table III.

TABLE III.—*Effect of Fluoride on the Oxidation of* D-*β-hydroxybutyrate by Liver Mitochondria Incubated Together with Tumour Mitochondria*

Experimental conditions as in Table I. In this case 5 μ-moles D-BHB were added. 16 μ-moles NaF were present as indicated in the table.

Mitochondria from	N present per flask (mg.)	Oxygen uptake (μ-atoms)	Acetoacetate formation (μ-moles)
Liver	1·52	10·1	4·0
Liver+T17572	1·52+0·97	0·0	—
Liver+T17572+DPN	1·52+0·97	5·2	4·5
Liver+T17572+F	1·52+0·97	4·9	4·4

The nature of the fluoride effect would thus involve a direct inhibition of the tumour mitochondrial DPNases. However, some evidence accumulated (Emmelot

and Brombacher, 1956*a*) that the effect of fluoride could also be explained on account of its potent inhibitory action on the tumour mitochondrial " ATPases ": by protecting the " high-energy " phosphate fluoride would indirectly stabilize the biochemical integrity of the mitochondria and thus counteract the development of the DPNases. Table IV illustrates that in the combined liver-tumour mitochondrial system with fluoride present, a net synthesis of ATP did indeed take place during the oxidation of DL-BHB.

TABLE IV.—*Effect of Fluoride on the Oxidative Phosphorylation of Liver Mitochondria Incubated Together with Tumour Mitochondria*

This experiment belongs to the series illustrated in Table II. Sixteen μ-moles NaF were present.

Mitochrondria from	DL-BHB	P_i found (μ-moles)	$-\Delta P_i$ (μ-moles)	$-\Delta O$ (μ-atoms)	P : O
Liver+T17572+F	Absent	14·9	3·7	2·8	1·3
	Present	11·2			

It might now be expected that fluoride would also counteract the inhibition of the oxidation of L-BHB in the combined liver-tumour mitochondrial system. However an effect of fluoride on this oxidation was difficult to demonstrate: only in a few experiments a small oxidation was found. This discrepancy may be understandable in view of the inhibitory effect of fluoride on certain Mg^{2+}-dependent reactions. Although by its same virtue, fluoride inhibits the ATPase and thus protects the ATP necessary for the formation of the L-BHByl-CoA bond, it might also easily inhibit the same reaction which is Mg^{2+}-dependent. This action of fluoride explained some of our earlier findings regarding the oxidation of octanoate (Emmelot and Bos, 1955*a*); Aisenberg and Potter (1956) have recently provided direct evidence for the inhibitory effect of fluoride on the formation of acetyl-CoA from acetate by rat liver mitochondria.

Phosphate release from ADP and ATP catalyzed by liver and tumour mitochondria

Inspection of the data listed in Column 4 of Table II shows that the concentration of P_i at the end of the 15 minutes' incubation of liver plus tumour mitochondria was higher (18 μ-moles) than that at the start of the experiment (14·4 μ-moles). Any extra phosphate must have been derived from the only other phosphate compound present, i.e. ADP, since in control experiments mitochondria incubated in the absence of ADP did release only an insignificant amount of P_i.

The P_i release from ADP was investigated in more detail by incubating sucrose-EDTA-prepared tumour mitochondria with ADP in the absence of oxidizable substrate in the Tris-KCl-$MgCl_2$ medium of Chappell and Perry (1954). Earlier experiments had established that the " ATPases " of mitochondria isolated from different tumour strains were not uniformly active: they varied from the order shown by liver mitochondria to higher values (Emmelot, Bos and Brombacher, 1956). Table V shows that the particles followed the same relative order of activities in releasing P_i from ADP. Very pronounced activities were inherent in that group of tumour mitochondria which uncoupled the oxidative phosphorylations of the liver mitochondria.

Since the breakdown of ADP to P_i takes place through the combined action of the two mitochondrial enzymes, myokinase (adenylate kinase) and ATPase

TABLE V.—*Phosphate Release from ADP Catalyzed by Tumour Mitochondria*

Mitochondria isolated in 0·25 M sucrose—0·001 M EDTA. Incubation at 27° C. in the Tris-KCl-$MgCl_2$ medium of Chappell and Perry (1954).

Mitochondria from	N present per flask (mg.)	μ-moles P_i/mg. N released after minutes			
		5	10	20	30
T17572 (adrenal cortex carcinoma)	0·28	10·0	18·3	22·8	28·1
	0·23	11·6	18·0	25·3	30·0
UV256 (sarcoma)	0·20	5·5	9·7	17·7	24·2
T26473 (hepatoma)	0·25	6·1	10·3	17·6	23·1
BY256 (rat adenohepatoma)	0·21	5·6	10·2	18·3	24·6
T5441 (granulosa cell tumour ovary)	0·21	2·0	3·0	5·5	7·0
T26554 (interstitial cell carcinoma testis)	0·21	2·3	3·8	7·3	10·4
Liver (mouse)	0·25±0·03	0·0–1·5	1·2–2·4	2·0–3·9	4·0–6·0
Liver (rat)	0·22	0·0	1·8	3·0	5·4

(2ADP → ATP + AMP; ATP → ADP + P_i, etc.) both activities appear to be high in these tumour mitochondria, which have been shown earlier to possess also markedly active DPNases and TPNases (Emmelot and Brombacher, 1956*b*).

The group of tumour mitochondria that had been found in the earlier experiments to be the most intact from the biochemical point of view, released very much less P_i from ADP as illustrated by two representative experiments (testicular tumour T26554 and ovarian tumour T5441) in Table V. These mitochondria did not inhibit the fatty acid oxidation of the liver mitochondria and possessed smaller DPNase and TPNase activities than the former tumour mitochondria (Emmelot, Bos and Brombacher, 1956).

A competition for the ATP, formed from ADP by the myokinase, may be expected between the "ATPases" of the tumour mitochondria and any other "high energy" phosphate trapping system. As shown in Table VI, the amount of P_i released from ADP is smaller the higher the concentration of hexokinase that was added together with a constant quantity of glucose. Hexokinase concentrations higher than those listed in the table did not alter the results very much, which indicated that the "ATPases" had a far more ready access to the ATP, resulting from the myokinase action on ADP, than the soluble hexokinase.

TABLE VI.—*Phosphate Release from ADP Catalyzed by Tumour Mitochondria in the Absence and Presence of Hexokinase-glucose*

Experiment 1: Mitochondria from UV256 sarcoma.
Experiment 2: Mitochondria from T17572 adrenal cortex carcinoma.
Glucose 0·025 M, hexokinase as indicated.

Experiment No.	Hexokinase (Kunitz-McDonald units)	μ-moles P_i/mg. N after minutes			
		5	10	20	30
1	0	5·3	9·4	16·5	23·3
	14	4·4	8·2	14·6	18·0
	35	2·8	7·0	12·5	16·0
2	0	—	16·1	27·0	—
	28	—	10·1	19·2	—
	35	—	10·0	18·2	—

Sucrose-prepared mitochondria from mouse and rat liver acting upon ADP, while suspended in the Tris-KCl-$MgCl_2$ medium, were found to release a very

small quantity of P_i from the nucleotide. The ranges of activity calculated per mg. mitochondrial nitrogen for 5 experiments with mouse liver particles are given in Table V. In a similar experiment conducted in isotonic sucrose with rat liver mitochondria by Siekevitz and Potter (1953) no P_i was found. Kielly and Kielly (1951), Potter, Simonson and Siekevitz (1953) and others have shown that the " ATPase " activity of freshly-prepared mouse and rat liver mitochondria suspended in isotonic sucrose is extremely low or completely absent (latent " ATPase "). The " ATPase " of the fresh mouse liver mitochondria under the present experimental conditions were not completely latent as shown in Fig. 1 (solid lines) but agreed well with the findings of others (Wade and Jones, Jr., 1956).

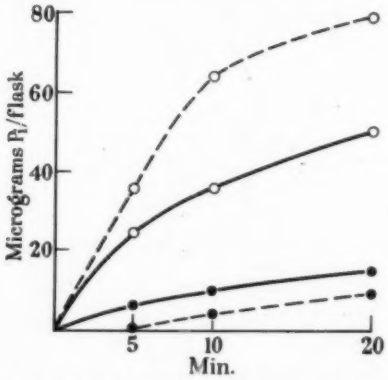

FIG. 1.—The effect of γ-globulin on mouse liver mitochondrial ATPases and their activation by 2 : 4 DNP.

Isolation of mitochondria in 0·25 M sucrose, incubation at 27° C. in Tris buffer (0·05 M) containing KCl (0·1 M), $MgSO_4$ (0·005 M) and ATP (0·005 M). pH 7·4. 0·05 mg. mitochondrial N per flask.

●——● controls, ○——○ 2 : 4-DNP (10^{-4} M) present, ●- - -● γ-globulin (2 per cent) present, ○- - -○ γ-globulin + 2 : 4-DNP present.

Under the conditions of the earlier experiments (Emmelot, Bos and Brombacher, 1956) we were unable to find any activation of the tumour mitochondrial " ATPases " in the presence of 2 : 4-DNP and only a two-fold activation of the corresponding activities of the liver mitochondria was observed. The 2 : 4-DNP activation has now been studied with small amounts of sucrose mitochondria and using γ-globulin to afford some protection against the degenerative changes which are suffered by the mitochondria *in vitro*. In experiments with the sucrose mitochondria from 25 mg. of mouse liver, 2 per cent γ-globulin was found to suppress the spontaneous activation of the latent " ATPases " as shown in Fig. 1 (compare Wade and Jones, Jr., 1956). It has further been observed that 2 : 4-DNP (10^{-4} M) activates the " ATPases " of our liver preparations to a greater extent in the presence of γ-globulin than in its absence (Fig. 1).

The active ATPases of the sucrose-EDTA mitochondria from the adrenal cortex carcinoma T17572 could be also suppressed by γ-globulin but only during the first 10 minutes of incubation (Fig. 2). 2 : 4-DNP (10^{-4} M) did activate the tumour mitochondrial ATPases under the latter conditions and caused a phosphate

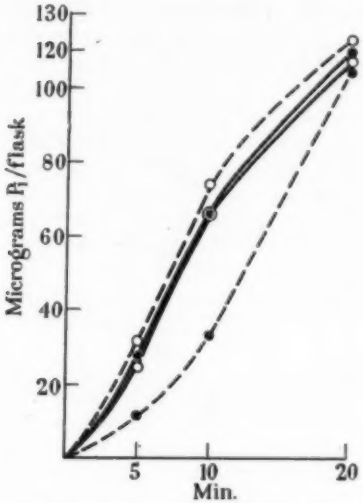

FIG. 2.—The effect of γ-globulin on tumour mitochondrial ATPases (mouse adrenal cortex carcinoma T 17572) and their activation by 2 : 4-DNP.
Isolation in 0·25 M sucrose containing 0·001 M EDTA (pH 7·4) 0·05 mg. mitochondrial N per flask. Compare Fig. 1.

release which was of the same order as that shown by the mitochondria in the absence of the protective protein. The less active ATPases of the sucrose-EDTA mitochondria from the hepatoma T28012 were markedly lower in the presence of γ-globulin during the whole 20-minute incubation period. 2 : 4-DNP activated the ATPases to a marked extent under the latter conditions (Fig. 3).

An unexpected stimulating effect on the mitochondrial ATPases was found when the suspension medium was supplemented with 2 per cent aged human serum albumin. This preparation had been kept in our laboratory for some five years at room temperature. The aged albumin was found to activate the liver mitochondrial ATPases in the same manner as 2 : 4-DNP. The activation of the tumour mitochondrial ATPases was especially significant after the first 5 minutes and decreased in proportion when the incubation was carried out longer (Fig. 4). 2 : 4-DNP did not activate the ATPases of the liver or tumour mitochondria further when the aged protein was present. The effect of the preparation is contrary to the protection that may be expected to arise from the addition of fresh albumin and that was indeed also found. When incubated separately with ATP the aged albumin did not cause P_i release and neither did it affect the pH of the suspension medium during the incubation. The latter was rigorously controlled since the ATPase activities of the tumour mitochondria, like those of liver mitochondria (Swanson, 1956), have been found (unpublished observations) to vary with the pH of the medium. After cold extraction with petrol-ether the aged albumin still exhibited its effect. The extract was evaporated but the small amount of fatty residue did not produce any activation of the ATPases of the liver mitochondria.

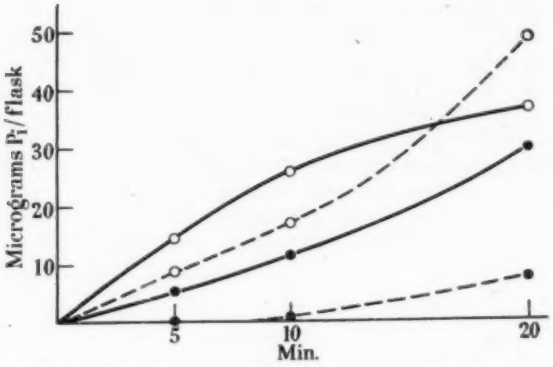

FIG. 3.—The effect of γ-globulin on tumour mitochondrial ATPases (mouse hepatoma T 28012) and their activation by 2 : 4-DNP.

Isolation in 0·25 M sucrose containing 0·001 M EDTA (pH 7·4) 0·04 mg. mitochondrial N per flask. Compare Fig. 1.

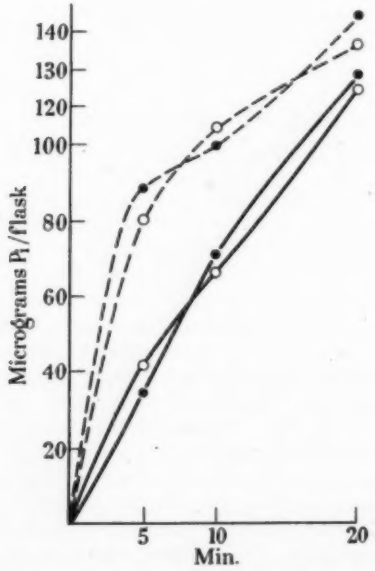

FIG. 4.—The effect of aged albumin on tumour mitochondrial ATPases (mouse adrenal cortex carcinoma T 17572) and their activation by 2 : 4-DNP.

Isolation in 0·25M sucrose containing 0·001 M EDTA (pH 7·4) 0·12 mg.N per flask.

●——● controls, ○——○ 2 : 4-DNP (10^{-4} M) present, ●- - -● aged albumin present (2 per cent), ○- - -○ aged albumin plus 2 : 4-DNP present.

Phosphate release from ADP and ATP catalysed by mitochondrial sub-fractions

Myokinase can be obtained in a soluble form by disrupting liver mitochondria (Kielley and Kielley, 1951, 1953), while the "ATPase" remains bound to the sedimentable mitochondrial débris. Any appreciable P_i release from ADP will thus only be possible when the soluble and sedimented parts of the disrupted liver mitochondria are incubated together. This is clearly illustrated by the experiments listed in Table VII which were carried out with mouse liver mitochondrial sub-fractions obtained by disrupting the mitochondria, as described under Material and Methods, and centrifugation for 30 minutes at 20,000 × g at pH 7·4. Centrifugation at pH 5·0 did not change the results. The synergistic action between the two sub-fractions in liberating P_i from ATP may be readily explained on account of the myokinase activity of the soluble fraction which removes the ADP that is inhibitory to the "ATPase" (Kielley and Kielley, 1953).

Table VII.—Phosphate release from ADP and ATP Catalyzed by Liver Mitochondrial Sub-fractions.

1 = sedimented fraction and 2 = soluble fraction of disrupted mitochondria ; 1 + 2 = fractions combined after separation. In Experiments *a* and *b* centrifugation was carried out at pH 7·4, in Experiment *c* at pH 5·0 ; compare under Materials and Methods. Experiments *b* and *c* were performed with the same batch but not the same amount of disrupted mitochondria.

Experiment	Substrate	µg. P_i released per flask after 20 minutes (by fraction)			mg. N/flask	
		1	2	1+2	1	2
a	ADP	31	6	104	0·27	0·10
	ATP	102	16	165		
b	ADP	10	8	125	0·16	0·16
	ATP	155	32	232		
c	ADP	10	4	98	0·26	0·16
	ATP	127	10	200		

Similar experiments were also carried out with the sub-fractions prepared from the tumour mitochondria. The first three experiments listed in Table VIII show that no synergistic action in liberating P_i either from ADP or ATP could be observed on mixing the two sub-fractions. This was apparently due to the lack of separation since the myokinase came down with the sedimented fraction. This occurred irrespective of the fact whether the centrifugation was carried out at pH 5·0 or 7·4. After centrifugation at 7·4 the supernatant was somewhat turbid. The pH was therefore changed to 5·0 ; this resulted in a clear supernatant.

Judging from the nitrogen content of two sub-fractions it appeared also that only a small part of the particulate material became soluble in the case of the tumour mitochondria.

Evidently, either the tumour mitochondria contained less soluble material or the disruption was far from complete. The latter may not be unreasonable since the size of the tumour mitochondria was usually smaller than that of the liver mitochondria. Therefore hepatoma mitochondria were used which were approximately equal in size to the liver mitochondria. In this case (T28012)

TABLE VIII.—*Phosphate Release from ADP and ATP Catalyzed by Tumour Mitochondrial Sub-fractions*

(Compare Table VII)

Experiment *a*: Mitochondria isolated in sucrose-EDTA. Separation of sub-fractions by centrifugation at pH 7·4; Experiment *b*: *ibidem* at pH 5·0; Experiments *c* and *d*: mitochondria isolated in the absence of EDTA, separation of sub-fractions at pH 5·0; Experiments *e* and *f*: same batch of mitochondria, isolated in sucrose-EDTA, separation of sub-fractions at pH 5·0; during the treatment with distilled water 10^{-4} M Na-dodecyl-sulphate was present in Experiment *f*.

Experiment	Mitochondria from	Substrate	μg. P_i released per flask after 20 minutes (by fraction) 1	2	1+2	mg. N/flask 1	2
a	T5358 (testicular carcinoma)	ADP	71	22	92	0·15	0·05
		ATP	140	26	157		
b	T17572 (adrenal cortex carcinoma)	ADP	132	0	141	0·23	—*
		ATP	176	0	180		
c	R733 (ovarian tumour rat)	ADP	125	2	143	0·14	0·03
		ATP	200	3	212		
d	T28012 (hepatoma)	ADP	52	5	85	0·21	0·04
		ATP	112	5	119		
e	„ „	ADP	35	9	62	0·12	0·02
		ATP	88	20	116		
	„ „	ADP	32	3	72	0·16	0·08
		ATP	120	32	152		

* Sample lost.

not withstanding the small amount of soluble nitrogen some synergism was found between the two sub-fractions in liberating P_i from ADP but not such an effect was seen with ATP. The results of the latter experiment could be readily reproduced with other tumours of the same strain but the mitochondria from another hepatoma (T26473) yielded negative results.

The final elucidation of this problem awaits further investigation of tumour mitochondria disrupted by other means and the application of higher centrifugal forces.

Our type of liver mitochondrial sub-fractions was apparently different from that of Keilley and Kielley (1953). These authors found that on disrupting liver mitochondria in a Waring blendor, about 60–70 per cent of the ATPase activity released was recovered in the supernatant after centrifugation at 20,000 × g and that 80–90 per cent of this activity was in turn recovered in the pellet after centrifugation at 110,000 × g. Since our ATPase preparation from the disrupted liver mitochondria was almost completely sedimentable at 20,000 × g, it is evident that in the present work the mitochondria were not broken down to such small fragments as in the experiments of the American authors.

Oxidative phosphorylation by tumour mitochondria

It has not been possible to obtain satisfactory oxidation of BHB by the tumour mitochondria which show high ATP- and DPN-splitting activities (sarcoma

UV256, adrenal cortex carcinoma T17572, etc.) The only possible exception is provided by the mitochondria from the hepatoma T26473 which may be classified however on other grounds as the least representative of this group of tumours (Emmelot and Bos, 1955*a*, 1956*b*).

Most of the other tumour mitochondria exhibiting smaller ATP- and DPN-splitting activities than the former, show a satisfactory oxygen uptake in the presence of D-BHB but are unable to oxidize L-BHB. This oxidation of D-BHB, like that of citric acid cycle intermediates as shown for other tumour mitochondria (Williams-Ashman and Kennedy, 1952; Kielley, 1952; Lindberg *et al.*, 1953; Potter and Siekeviz, 1951) is accompanied by phosphorylation. In the absence of fluoride low P : O ratios, but in the presence of fluoride (0·01 M) P : O ratios of over 2 were obtained as illustrated in Table IX. The action of fluoride in inhibiting the tumour mitochondrial myokinase and "ATPase", which enzymes would otherwise make any net phosphate uptake difficult to demonstrate, is very clearly illustrated in the experiment with the mitochondria from T26473 (compare Table V). Some control experiments on the effect of fluoride on the phosphate release from ATP and ADP by tumour mitochondria over a 15-minute period did indeed show that the "ATPases" were inhibited for 50-60 per cent and the myokinases for 80–100 per cent.

In a few cases the mitochondria from the sarcoma UV256 effected a small oxidation of pyruvate in the presence of L-malate and DPN. The P : O ratio found was half of that obtained with the mitochondria from the ovarian tumour T5441 or the testicular tumour T26554. This again demonstrated the difference in biochemical integrity between the representative of the two groups of tumour mitochondria.

TABLE IX.—*Phosphorylation Accompanying the Oxidation of* DL-*BHB and Pyruvate by Tumour Mitochondria*

Experimental conditions as in Table II, except that tumour mitochondria (approximating 0·5–0·7 mg. N) 21 μ-moles P_i and 16 μ-moles NaF were present. In four experiments 2 μ-moles pyruvate and 0·5 μ-mole L-malate were added.

Mitochondria from	Substrate	$-\Delta P_i$ μ-moles	$-\Delta O$ μ-atoms	P : O
T 5358 (testicular carcinoma)	DL-BHB	4·3	1·9	2·3
T26473 (hepatoma)	„	6·9	2·4	2·9
T28102 (hepatoma)	„	7·7	3·7	2·1
T 5441 (ovarian tumour)	Pyruvate+L-malate	4·0	2·5	1·6
UV 256 (sarcoma)	„ „	1·2	1·5	0·8
T26554 (testicular tumour)	„ „	10·7	5·8	1·8
CBA71 (hepatoma)	„ „	10·8	5·4	2·0

SUMMARY

It has been shown that sucrose-EDTA prepared mitochondria from certain tumour strains uncouple the phosphorylations normally associated with the oxidation of D-β-hydroxybutyrate by liver mitochondria, when both types of mitochondria are incubated together in the presence of DL-β-hydroxybutyrate and DPN. Consequently, the ATP-dependent oxidation of L-β-hydroxybutyrate, and in analogy that of octanoate, is abolished in a combined liver-tumour mitochondrial system. The tumour mitochondria possessed very pronounced "ATP-

ase " and myokinase activities as shown by the release of inorganic phosphate from ADP. In contrast, mitochondria from a number of other tumours, which have been shown earlier to be biochemically more intact than the former mitochondria (e.g. " ATPases ", DPNases and TPNases less active and no inhibition of the ATP- or DPN-dependent oxidative processes of the liver mitochondria) released much less inorganic phosphate from ADP. The latter tumour mitochondria yielded P : O ratios from 2–3 in the presence of DL-β-hydroxybutyrate or pyruvate when fluoride was added.

Myokinase and " ATPase " activities could easily be separated by disruption of liver mitochondria and centrifugation but in the case of tumour mitochondria results were unsatisfactory.

Addition of 2 per cent fresh human serum γ-globulin suppressed the " ATPase " activities of both liver and tumour mitochondria. Under these conditions an activation of the tumour mitochondrial " ATPases " by 2 : 4-dinitrophenol could readily be demonstrated. An aged preparation of human serum albumin, unlike fresh albumin, activated the enzymes of both types of mitochondria very significantly.

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